Anti-T and anti-B cell therapy in renal transplantation

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Chapter 1

Outline of the thesis

In most transplant centers immunosuppressive therapy after renal transplantation consists of the combination of a calcineurin inhibitor, mycophenolic acid and steroids, combined with induction therapy. With this treatment the incidence of allograft rejection during the first six months after transplantation has dropped to 10-20% in the last decades. This has greatly improved allograft and patient survival in the first year after transplantation [1]. Prevention of allograft rejection does also reduce the risk of chronic allograft dysfunction, which is the cause of late graft loss in about half of the patients. By further reducing the incidence of acute allograft rejection, long-term graft survival could therefore improve. Although strong immunosuppressive therapy can result in lower rates of acute allograft rejection, this benefit is almost always counterbalanced by an increased risk of infections and cancer. The optimal therapy to prevent rejection should therefore effectively intervene with the immune response against the allograft, while leaving the rest of the immune system as competent as possible to fight micro-organisms and malignant cells.

Another strategy to optimize results after transplantation is the safe and effective treatment of severe allograft rejection. If allograft rejection persists despite steroid-therapy, additional therapy is required to prevent graft loss. The current treatment with polyclonal anti-T cell immunoglobulins is effective but associated with severe side effects, which limits its use. The challenge is to find a treatment that is at least as effective to revert the rejection process, but is better tolerated.

A third important issue after transplantation is the occurrence of delayed graft function. This results in the prolonged need for dialysis, with increased morbidity, mortality, and prolonged hospital stay. Moreover, the lack of early graft function requires performing graft biopsies at regular intervals to exclude acute allograft rejection. The incidence of delayed graft function is especially high after transplantation of kidneys donated after circulatory death (DCD), since in these cases there is an additional warm ischemia time which is lacking in donation after brain death (DBD).

This thesis is based on studies concerning the safety and efficacy of novel approaches to address these three issues (prevention of rejection, treatment of steroid-resistant rejection and prevention of delayed graft function). As an introduction for the clinical trials presented in this thesis, **chapter 2** describes the general aspects of allograft rejection and its treatment with anti-T cell antibodies. In **chapter 3** the place of monoclonal antibodies in renal transplantation is described.

In **chapter 4** the high incidence of delayed graft function that occurs after kidney donation after circulatory death is discussed. We investigated if combining T cell depletion (with ATG-Fresenius) with a regular, unadjusted dose of tacrolimus would lead to a reduced incidence and duration of delayed graft function after transplantation with a DCD donor kidney.

In **chapter 5** the issue of steroid-resistant allograft rejection is addressed. The current standard treatment for steroid-resistant allograft rejection is the polyclonal anti-T cell preparation ATG-Thymoglobulin. An alternative agent that can be used for the treatment of these rejections is alemtuzumab, a monoclonal antibody that depletes T and B cells. In a retrospective analysis we compared the effectiveness and safety of ATG-Thymoglobulin and alemtuzumab for the treatment of steroid-resistant allograft rejection.

While immunosuppressive therapy traditionally focuses on T cells, the role of B cells and antibodies in acute allograft rejection has received more attention over the recent years for several reasons. First because of the negative prognostic impact of donor-specific anti-HLA antibodies. Secondly because of the presence of B cell clusters in biopsies of patients with severe rejection, and thirdly because of the frequent finding of capillary deposition of C4d, a split product of C4 of the classical complement pathway, in patients with acute rejection [2, 3]. B cells are the progenitors of plasma cells, they function as effective antigen presenting cells, and they can secrete different cytokines to stimulate the immune system. Moreover, interfering specifically with B cells by rituximab treatment has shown to be effective in diseases that were considered to be mainly T cell driven, like rheumatoid arthritis [4, 5].

For these reasons, B cell depletion at the time of transplantation might be beneficial to prevent acute allograft rejection. In **chapter 6** the results are reported of a randomized, placebo-controlled clinical trial in 280 renal transplant patients treated with either a single dose of rituximab or placebo at the moment of transplantation, added to a standard immunosuppressive regimen. We tested the hypothesis that this would safely and effectively reduce the incidence of biopsy-proven acute renal allograft rejection during the first six months posttransplant.

Chapter 7 focuses on the histopathology of acute allograft rejection in patients participating in the abovementioned clinical trial. Several researchers have reported an association between the presence of intragraft B cells during allograft rejection and a worse graft outcome. In this chapter we analyzed whether B cell depletion with

rituximab had an effect on the type of rejection (T cell mediated versus antibody mediated), and on the intragraft presence of B cells. We also examined whether the presence of B cells was associated with more steroid-resistance and a worse graft function

Since the immune system is a complex interplay between cells, tissues and molecules we explored whether rituximab had other effects in addition to B cell depletion. **Chapter 8** assesses the potential problem of the release of cytokines after infusion of rituximab, as is seen in lymphoma patients with high B cell counts. We studied this phenomenon in our transplant patients and tried to identify the cell type responsible for the cytokine release.

In **chapter 9** the effect of rituximab on IL-17 production is analyzed. Th17 cells are characterized by the ability to produce several cytokines (especially IL-17 and IL-21). Th17 cells are not only involved in the immune response against extracellular pathogens, but recent studies show that they might also play a role in allograft rejection and that B cells could contribute to a Th17 response. Based on these findings, we tested the effect of rituximab on the ability of T cells to produce cytokines (including IL-17) after ex vivo stimulation.

Finally, in **chapter 10**, the findings from the abovementioned studies are summarized and put into perspective of the current and future practice of renal transplantation.

Chapter 2

Anti-T cell antibodies for the treatment of acute rejection after renal transplantation

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Abstract

Introduction Given the central role of T cells in the alloimmune response, anti-T cell antibodies retain a prominent place in the treatment of renal allograft rejection. During the past decades, many anti-T cell antibodies have emerged and subsequently left the field of solid organ transplantation, but rabbit antithymocyte globulin (ATG) and the anti-CD52 humanized rat monoclonal antibody alemtuzumab have remained.

Areas covered This article reviews the literature about the use of ATG and alemtuzumab for the treatment of acute rejection after renal transplantation. Furthermore, it discusses possible side effects, including infusion reactions. A literature search using PubMed and Embase databases was undertaken using search words alemtuzumab, antithymocyte globulin, rejection, kidney and renal transplantation.

Conclusion Treatment of severe or steroid-resistant renal allograft rejections with ATG is very effective, but is also associated with frequent infusion reactions and an increased incidence of infections and posttransplant lymphoproliferative disease. Alemtuzumab may prove to be an attractive alternative. It can be administered easily, is relatively cheap and nearly devoid of acute side effects, but the long-term efficacy and safety as antirejection treatment are currently difficult to judge.

The immunology of rejection

Next to the surgical challenges of transplantation, the manipulation of the alloimmune response is crucial for a successful renal transplantation. The alloimmune response consists of a recognition phase and an effector phase, in which different cells and molecules are involved [6]. After reperfusion, the immune system of the recipient comes into contact with alloantigens of the donor. Incompatibility between donor and recipient leads to an immune response. The non-self-HLA (human leukocyte antigen) molecules of the donor are the major alloantigens, although disparities in minor alloantigens (e.g., the aldosterone receptor or HY antigen) also play a role in the alloimmune response [7]. The alloantigens are processed by antigen-presenting cells expressing MHC (major histocompatibility complex) class II molecules, such as dendritic cells, macrophages and B cells. Hereby, they stimulate naive or memory T cells either in secondary lymphoid organs or in the graft itself. The T cells recognize a specific part or peptide of the processed alloantigen in the context of the MHC molecule via their T cell receptor (TCR). Like T cells, B cells can be activated when they recognize complete, unprocessed alloantigens with their B cell receptor.

After binding of the alloantigen to the TCR, a second, costimulatory signal has to be provided by the antigen-presenting cell for optimal T cell stimulation. This provides the initiation of a cascade of different molecular pathways which results in increased transcription of many proteins, including IL-2. Binding of IL-2 to its receptor activates a pathway in which the molecular target of rapamycin is stimulated. This finally leads to progression of the cell cycle and cell proliferation and differentiation, hereby starting the effector phase.

In the effector phase, naive CD4+ T cells can differentiate into specific T cell subsets. Initially, there were thought to be only two major subsets; T-helper 1 (Th1) cells, that are mainly involved in cell-mediated responses, and T-helper 2 (Th2) cells, especially providing help in humoral immunity. Afterwards, new subsets have been described, such as regulatory T cells (Treg) which preserve peripheral tolerance, and T cells that secrete IL-17 (Th17).

Most B cells need T cell help for full activation, affinity maturation and immunoglobulin class switching. After differentiation into plasma cells, alloantibodies are produced, of which IgM or immune-complexed IgGs in particular can lead to classical pathway activation of the complement system. This activation will result in cleavage and activation of a cascade of proteins, finally resulting in the formation of the membrane

attack complex which leads to endothelial cell damage. Cytotoxicity via this mechanism is known as complement-dependent cytotoxicity. The Fc part of an antibody can also bind to Fc receptors on natural killer (NK) cells and myeloid cells. Once the Fc receptor binds to the Fc part of the antibody, the effector cell releases cytokines and the contents of cytotoxic granules (perforin and granzymes) resulting in cell death by triggering apoptosis (antibody-dependent cell-mediated cytotoxicity). The extent to which complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity take place depends on the density of bound antibodies, and the class and subclass of the antibodies [8].

Histopathological changes during acute rejection

The histopathological hallmark of acute renal allograft rejection is massive influx of mononuclear cells (mainly T cells, but influx of B cells, monocytes, macrophages and mast cells can also be seen) in the kidney parenchyma. This leads to tubulointerstitial damage (especially tubulitis) and intimal arteritis. When this inflammation is not halted or the antigen is not eradicated (which is the case in renal transplantation), chronic inflammation can occur, which eventually will lead to chronic rejection and graft loss. In the chronically inflamed renal allograft, the immune cells can sometimes organize themselves into structures that resemble lymphoid organs, so-called lymphoid neogenesis. The infiltrate is not only composed of proliferating T cells, but also of B cells which contribute to a local humoral alloimmune response [9]. To ensure standardized reporting on the examination of renal allograft biopsies, the Banff 97 working classification of renal allograft pathology is used. This classification is based on semiquantitative lesion scoring focusing on tubulitis and arteritis, and is universally accepted in the transplant society [10].

A humoral or antibody-mediated rejection in renal allografts is characterized by the demonstration of donor-specific anti-HLA antibodies in combination with certain histological features. Anti-HLA antibodies can bind to the renal vascular endothelium and lead to activation of the classical pathway of the complement system, causing deposition of complement split products such as C4d and C3d on the vascular endothelium. Activation of the complement system leads to the distinct pathological changes of humoral rejection, consisting of endothelial injury with fibrinoid changes in the vessel walls, thrombosis, glomerulitis and especially accumulation of polymorphonuclear leucocytes in peritubular capillaries [10]. In fact, renal allograft

rejections are seldom exclusively cellular or humoral, but in most cases components of both can be found on histopathological examination.

Anti-T cell antibody products

Given the central role of T cells in the alloimmune response, most efforts to control this response with antibodies have been directed toward the development of various kinds of depleting anti-T cell antibodies. Most experience has been gained with the use of polyclonal antibodies. These antibodies are derived from unfractionated serum after immunization of different animals, mainly rabbits and horses, with human lymphoid cells or tissue. Experience with monoclonal anti-T cell antibodies is more limited. During the past decades, many depleting anti-T cell antibodies have emerged and subsequently left the field of solid organ transplantation, like the monoclonal anti-CD3 antibody Muromonab-CD3 (Orthoclone OKT3) and horse antithymocyte/antilymphocyte globulin (ATG/ALG; ATGAM and ALG-Merieux). For the treatment of acute rejection, OKT3 and ATGAM/ALG-Merieux are both outperformed by ATG, which is at least as effective and has a somewhat more favorable side effect profile [11-13]. The declining usage of OKT3 and ATGAM/ALG-Merieux has led to their removal from the market.

To date, only three anti-T cell antibody products remain for use in clinical organ transplantation; rabbit-ATG from Genzyme (Cambridge, MA, USA), rabbit-ATG from Fresenius (Bad Homburg, Germany) and the anti-CD52 humanized rat monoclonal antibody alemtuzumab from Genzyme. The mechanism of action of these antibodies is not completely known, although induction of apoptosis, complement-mediated cell death and antibody-dependent cell cytotoxicity are the proposed mechanism of action [14].

Both ATG products are polyclonal antibodies and target a broad range of molecules and cells. ATG targets mainly T cell derived antigens, including CD2, CD3, CD4, CD8, CD25, CD28, CD45 and CD40L, but also CD16 (NK cell marker), CD11a, CD18 (leukocyte adhesion molecules) and even markers on B cells and plasma cells [15-17]. The broad immunomodulatory activity of ATG preparations is a consequence of the manufacturing process, during which unfractionated human thymocytes, and not only CD3+ T cells, are administered as immunogens.

ATG should be administered intravenously using a central venous catheter, high flow vein or arteriovenous fistula. Extravasation can lead to local inflammatory reactions. As rapid administration is associated with more acute infusion reactions (mainly pulmonary

edema, fever and hypotension), infusing the initial dose over 4 – 6 h helps to prevent or ameliorate these reactions. The duration of therapy is mainly dependent on center-specific preference, but is generally accepted to be 10 – 14 days. Close monitoring of the level of T cell numbers or total lymphocyte number in peripheral blood can be used to determine the dosage required to achieve T cell depletion and to limit (infectious) complications of excessive immunosuppression [18-20]. In ATG-treated patients, T cell depletion generally lasts for about a year, with a slower reconstitution of naive cells as compared with memory T cells [15].

Alemtuzumab is a humanized monoclonal antibody, directed specifically to the CD52 molecule which is expressed on T, B, and NK cells, monocytes, macrophages and dendritic cells [21]. In general, the depletion of T cells caused by alemtuzumab is more prolonged compared with that induced by ATG, with T cell recovery to about only 50% of baseline levels at 36 months after administration [22]. Detailed studies of lymphocyte subsets have shown a long-lasting reduction in thymic output of CD4+ T cells [23, 24]. For a further description of alemtuzumab, we refer to chapter 3 of this thesis.

Treatment of first acute rejection episodes

The first-line treatment of established acute cellular allograft rejection is high-dose steroids. Rather surprisingly, the dose and duration of high-dose steroid therapy have not been studied in randomized trials. Several prospective, randomized trials with anti-T cell antibodies have been performed, but only four of them directly compared ATG with high-dose intravenous or oral steroids (**Table 1**) [25-28]. These trials were relatively small (20 – 100 patients) and had a variable period of follow-up, ranging from 12 – 48 months. Direct comparison of the outcome of these trials is complicated by significant differences in treatment regimen, maintenance immunosuppression, and treatment in the control group. Only one study showed superiority of ATG in the prevention of recurrent rejection (16 vs. 72% in steroid group, $p < 0.01$), although this trial might have been biased by using a relatively low dose of steroids in the control group and a subsequent high risk of recurrent rejection in that group [27]. This trial did not show a significant difference in 4-year graft survival (80 vs. 88% in steroid group). However, since recurrent rejection is a predictor for chronic transplant failure, the effect might be visible after longer follow-up. Another trial compared administration of ATG with high-dose oral steroids (standard antirejection therapy for that time; 200 mg/day with rapid tapering to 25 mg in 2 weeks). This trial did not show a significant effect on recurrent

rejection (56 vs. 71% in steroid group), but showed improved patient (98 vs. 84% in steroid group, p = 0.02) and graft survival (78 vs. 50% in steroid group, p = 0.002) after a follow-up period of 1 year [26]. The other two trials did not show superiority of ATG with regard to either recurrent rejection or patient and graft survival.

In a recent meta-analysis, the above-mentioned trials were combined and pooled with data from additional studies that used accessory treatment (e.g., irradiation) or other antibodies (OKT3 or ALG). These pooled data show a lower failure rate to reverse rejection (relative risk of 0.57, with 95% confidence interval (CI) 0.38 – 0.67) and reduced graft loss (censored for death with functioning graft; relative risk 0.74 with 95% CI 0.58-0.95) in patients treated with anti-T cell antibodies as compared with steroids, irrespective of the antibody preparation used [13]. However, the higher efficacy of anti-T cell antibodies is counterbalanced by an impressive side effect profile and high costs. Therefore, high-dose steroids remain the current first-line treatment of first rejection episodes, reserving anti-T cell antibodies for steroid-resistant rejections. It remains unclear however, whether more severe rejections (Banff IIA or IIB) should be treated initially with anti-T cell antibodies [29].

Only two trials have been published with the off-label use of alemtuzumab for treatment of first episodes of acute rejection after renal transplantation. The first trial described long-term results of a cohort of 15 patients who received intravenous alemtuzumab as first-line treatment of biopsy-proven acute rejection [30]. These patients were compared with a historical control group, which received steroids as initial therapy, followed by ATG if rejection persisted. All rejection episodes were successfully treated with alemtuzumab and only one recurrent rejection occurred. After treatment with alemtuzumab, 10-year patient survival was significantly lower than observed in the control group (60 vs. 88%, p = 0.02) due to an excess of early infection-associated death. Ten-year graft survival was not different in both groups (40% in alemtuzumab group vs. 52% in control group), although there was a trend to better graft function in the alemtuzumab group (serum creatinine 143 vs. 183 µmol/l, p = 0.06). In another trial, a subgroup of 11 patients was also treated with alemtuzumab as first-line antirejection therapy [31]. However, data were analyzed for all of the 40 included patients, hampering the interpretation of these data.

Treatment of recurrent or steroid-resistant rejection episodes

When treatment with steroids fails or a rejection quickly recurs, treatment with anti-T cell antibodies is a logical next step. In this setting, various studies have been performed, comparing one antibody with another (OKT3 with ATG or ALG), comparing different preparations (rabbit ATG vs. horse ATG), or different doses (low-dose OKT3 vs. high-dose OKT3). After the removal of ALG and OKT3 from the market, the results of these studies have become less relevant. Nonetheless, three small trials (21 – 60 patients) comparing OKT3 with ATG did not show any superiority of OKT3 with respect to rate of rejection reversal, and graft or patient survival (**Table 2**) [17, 32, 33]. In the same meta-analysis as mentioned above, pooling of the data from these three trials did not change this conclusion [13]. Experience with the use of alemtuzumab for treatment of steroid-resistant rejection is scarce and no randomized trials have been published on this subject. Most papers report on a few patients, merely demonstrating that recurrent or steroid-resistant rejection can be reversed with alemtuzumab [34-36]. Interpretation is difficult however, since treatment of alemtuzumab was combined with other immunosuppressive therapies like plasmapheresis.

Table 1. Prospective, randomized trials comparing anti-T cell antibodies with corticosteroids in treatment of first acute rejection episode after renal transplantation

Author and reference	Treatment allocation		Maintenance immunosuppression	Follow-up (months)	Outcome	
	Antibody	Steroids			Recurrent rejection	Graft survival
Shield et al. [25]	ATG (ATGAM) 15 mg/kg for 14 days (n = 10)	Methylprednisolone 1000 mg for 5 days (n=10)	Azathioprine (2-3 mg/kg/day) Prednisone (2 mg/kg/day, tapered to 0.5 mg/kg/day)	26	ATG; 1/10 Steroids; 5/10 (NS)	ATG; 9/10 Steroids; 9/10 (NS)
Hoitsma et al. [26]	ATG (local preparation) 2-7 mg/kg for 21 days (n = 50)	Oral prednisone 200 mg/day, tapered in 14 days to 25 mg/day (n=50)	Azathioprine (1.5-3 mg/kg/day) Prednisone (100 mg/day, tapered to 25 mg/day)	12	ATG; 56%* Steroids; 71%* (NS)	ATG; 78%* Steroids; 50%* (p = 0.002)
Theodorakis et al. [27]	ATG (Fresenius) 4 mg/kg for 7 days (n = 25)	Methylprednisolone 250 mg for 3 days (n=25)	Cyclosporine (50-150 ng/ml) Methylprednisolone (4-8 mg/day, withdrawal >6 months)	48	ATG; 4/25 Steroids; 18/25 (p < 0.01)	ATG; 22/25 Steroids; 20/25 (NS)
Glass et al. [28]	ATG (ATGAM) 15 mg/kg or ALG-Merieux (30 mg/kg) for 14 days (n =35)	Oral prednisone 3 mg/kg, tapered to 30 mg/day (n=27)	Azathioprine (150 mg/day) Prednisone (30-120 mg/day, tapered to 30 mg/day)	12	ATG/ALG; 24/35 Steroids; 12/27 (NS)	ATG/ALG; 61%* Steroids; 53%* (NS)

* absolute number of patients could not be extracted from the paper, NS = not significant

Table 2. Prospective, randomized trials evaluating different anti-T cell antibodies in treatment of recurrent or steroid-resistant rejection episodes after renal transplantation

Author and reference	Treatment allocation		Maintenance immunosuppression	Follow-up (months)	Outcome	
	Muromonab-CD3	ATG/ALG			Recurrent rejection	Graft survival
Midtvedt et al. [17]	OKT3 2.5-5 mg/day depending on T cell numbers, 10 days (n = 28)	ATG (Genzyme) 1-2 mg/kg/day depending on T cell numbers, 10 days (n = 27)	Cyclosporine (150-300 ng/ml, dose reduced to 70-150 ng/ml during ATG/OKT3 treatment) Azathioprine (2 mg/kg/day, tapered to 1 mg/kg/day) Prednisone (80 mg/day, tapered to 10 mg/day)	30	OKT3: 12/28 ATG: 14/27 (NS)	OKT3: 24/28 ATG: 23/27 (NS)
Hesse et al. [32]	OKT3 5 mg/day, 10 days (n = 11)	ALG-Merieux 0.5 ml/kg, 10 days (n = 10)	ALG induction (0.5 ml/kg, 2-7 days) Azathioprine induction (1-3 mg/kg/day, 2-7 days) followed by cyclosporine (300 ng/ml) Prednisone (250 mg/day, tapered to 7.5 mg/day)	3	OKT3: 1/11 ALG: 2/10 (NS)	OKT3: 11/11 ALG: 7/10 (NS)
Mariat et al. [33]	OKT3 2.5-5 mg/day, 10 days (n = 29)	ATG (Genzyme) 25-75 mg/day, 10 days (n = 31)	Cyclosporine (dose not stated) Azathioprine (dose not stated) Prednisolone (dose not stated)	17	OKT3: 28%* ATG: 38%* (NS)	OKT3: 23/29 ATG: 27/31 (NS)

* absolute number of patients could not be extracted from the paper, NS = not significant

There is one larger observational trial describing 40 patients (mentioned above), in whom alemtuzumab was given because of steroid-resistant rejections, or rejections equal to or more severe than Banff 1B. Graft survival was 74% after 1 year, with a patient survival of 95% [31]. The incidence of recurrent rejection episodes was not reported. Due to the non-comparative nature of this study, it is difficult to draw conclusions regarding the efficacy as compared with that of other treatment modalities.

Side effects of anti-T cell antibodies

Although highly effective, the use of anti-T cell antibodies is associated with several problems. Especially the first administration of these antibodies can be followed by a release of various cytokines, mainly IL-6 and TNF-a. Most studies investigating the possible source of these cytokines are performed in OKT3-treated patients. In this setting, these cytokines are thought to be released by a transient activation of T cells, before they undergo cell lysis, although it is also possible that monocytes or macrophages are the source of the cytokines [13, 15, 37]. The clinical manifestations of a cytokine release syndrome are fever, chills, headache, dyspnea, myalgia and hypotension. The release of cytokines probably contributes to diarrhea, pulmonary edema and intra-allograft thrombosis. Leukocytopenia and thrombocytopenia are also commonly encountered side effects. Patients receiving OKT3 were approximately three times more likely than patients receiving other antibodies to experience these side effects [13]. Data on the safety of alemtuzumab are mainly based on its use as induction agent. In earlier trials, intravenous administration of alemtuzumab was associated with infusion reactions in about 50% of patients, but most were generally mild [31]. In more recent trials, in which alemtuzumab was given subcutaneously, infusion reactions were rare or not reported [38]. Taken together, the amount of data concerning the severity and extent of infusion reactions in patients with acute rejection treated with alemtuzumab remains very limited.

Owing to the broad number of cellular targets of polyclonal antibodies, undesired cross-reactivities can lead to additional toxicity. In the past, the production of polyclonal antibodies was technically challenging, leading to differences in biological activity between different batches. However, a recent study evaluating the capacity of different anti-T cell preparations to bind to leukocyte antigens and the successive functional effects showed little batch-to-batch variation [39]. Since the polyclonal anti-T cell antibodies are non-human, treatment can be followed by the formation of xenogeneic

(antihorse or antirabbit) antibodies. These xenogeneic antibodies can diminish the efficacy after extended or repeated use and can also lead to anaphylaxis and serum sickness [40, 41]. This can be a reason to avoid repeated use of polyclonal antibodies, for example, for treatment of acute rejection episodes after subsequent transplantations. Another approach is epidermal testing of all patients, or measurement of pre-formed xenogeneic antibodies in serum [42, 43]. To measure the presence of antibodies against rabbit IgG, a rheumatoid factor test that is based on agglutination of rabbit IgG-coated particles can be used. Data on the formation of xenogeneic antibodies after treatment with alemtuzumab are conflicting. Since it is a humanized antibody, a low formation rate of xenogeneic antibodies would be expected. In 62 leukemia patients who were treated with alemtuzumab, only 2 patients (3%) produced significant levels of anti-alemtuzumab antibodies [44]. However, in patients with rheumatoid arthritis, anti-alemtuzumab antibodies could be found in the majority of alemtuzumab-treated patients (54% to even 100%) [45, 46]. Especially repeated, subcutaneous administration seemed to favor the formation of xenogeneic antibodies.

Treatment of multiple sclerosis patients with alemtuzumab is associated with an increased incidence of secondary autoimmune disease, especially of the thyroid gland [47]. Autoimmune thyroid disease after treatment with alemtuzumab has also been reported in renal transplant patients [48]. The mechanism of (thyroid) autoimmunity after alemtuzumab treatment is likely related to loss of self-tolerance in the immune reconstitution that occurs following profound lymphopenia, and in multiple sclerosis patients the risk is higher in patients with increased levels of IL-21 [49].

Infectious complications of anti-T cell antibodies

Patients who received anti-T cell antibodies appear more likely to develop serious and sometime fatal infections, especially if they have received a significant immunosuppressant load before treatment (either before or after transplantation) [30, 31]. Most experience stems from the use of anti-T cell antibodies as induction therapy. Of special interest is the reported increased incidence of cytomegalovirus (CMV) disease in early trials. In later trials, using adequate antiviral prophylaxis, an increased incidence after induction was not reported [15]. After rejection therapy with anti-T cell antibodies, no increased risk of CMV infection has been reported, either in individual trials or in pooled data, but this may have been accomplished by the use of anti-viral prophylaxis [13]. In one induction trial, patients treated with alemtuzumab showed less acute

rejections but an increased incidence of CMV disease [50]. This could not be confirmed in a larger cohort of 547 patients [51]. However, the latter study showed that the risk of developing opportunistic infections was increased when patients received alemtuzumab for the treatment of allograft rejection compared with those who received alemtuzumab as induction therapy (21 vs. 4.5%; $p < 0.001$). In a recent, non-comparative study with 40 patients treated with alemtuzumab for steroid-resistant or severe rejection, a total of 14 patients (35%) had infectious complications, of whom two (5%) died [31].

Malignant complications of anti-T cell antibodies

Another feared side effect of anti-T cell antibodies is the development of posttransplant lymphoproliferative disease (PTLD). The incidence of PTLD is probably higher after treatment with anti-T cell antibodies as compared with high-dose steroids [13, 52]. Because PTLD can occur after completion of the follow-up of clinical trials, most data are derived from registry databases. These databases have their specific drawbacks and cannot prove causality, but most of them show a slightly increased risk of PTLD when anti-T cell antibodies are used for induction. The Scientific Registry of Transplant Recipients performed an analysis of 41,686 patients who underwent transplantation [53]. A total of 181 patients (0.43%) developed PTLD and patients receiving anti-T cell antibodies as induction treatment had a relative risk of 1.8 (95% CI 1.3 – 2.4). This risk appeared to be especially high in ATG-treated patients, with a relative risk of 3.0 (95% CI 1.5 – 5.9). In the US Renal Data System, using a population of 38,519 patients, the relative risk of developing PTLD was only increased in patients receiving monoclonal antibodies for induction, with a relative risk of 1.7 (95% CI 1.0 – 2.8) [52]. A study based on 25,127 Medicare patients could not find any increment in any subcategory [54]. The risk of PTLD seems to decrease over time, with an actual incidence of below 0.5 – 1% at several years after transplantation. In a recent meta-analysis, no increase of the risk of PTLD or any other malignancy could be found in patients treated with anti-T cell antibodies for rejection, compared with steroids [13].

In clinical trials, only a few cases of PLTD have been reported after the use of alemtuzumab [55, 56]. This is possibly related to the potent depletion of both T and B cells. The largest survey has been performed using the Organ Procurement and Transplant Network/United Network for Organ Sharing (OPTN/UNOS) database [57]. In this analysis, 59,560 kidney recipients were studied, of which 1691 were treated with alemtuzumab as induction therapy. The incidence of PTLD within 2 years after

transplantation was 0.37%, which was not significantly different compared with either no induction therapy (0.43%), or induction with basiliximab (0.38%) or daclizumab (0.33%). In the same analysis, ATG was associated with a significantly increased risk of PTLD (0.67%).

Differences between ATG and alemtuzumab

The main differences between ATG and alemtuzumab are summarized in **Table 3**. ATG targets a broad range of T cell surface antigens, but it also contains antibodies against NK and B cells. Alemtuzumab targets a single antigen, which is found on different cells: T, NK, and B cells. Alemtuzumab induces a more profound depletion of B cells than ATG [58]. Moreover, CD52 expression has also been described for monocytes, macrophages, dendritic cells, eosinophils and mast cells [59-61]. Whether these differences affect the efficacy of the antibodies to combat graft rejection is currently unknown.

Both ATG and alemtuzumab can induce long-lasting depletion of T cells. In general, the depletion of T cells caused by alemtuzumab is more prolonged, with T cell recovery (especially CD4+ T cells) to only 50% of baseline levels at 36 months after administration [22-24]. Depletion of other cell types is variable; NK cells return within 1 month, monocytes within 2 months, and B cells usually return within 6 months [62]. In ATG-treated patients CD4+ T cell depletion generally lasts for about a year [15].

As stated above, the reported incidence of infusion reactions is lower in patients receiving alemtuzumab, compared with those who receive ATG. Alemtuzumab can be administered by subcutaneous injection with potential advantages regarding safety, flexibility and convenience [63]. The need for a central venous catheter, which is usually required for administration of ATG and is known to be a potential site of infection, is absent in case of treatment with alemtuzumab. In most cases, treatment with alemtuzumab is cheaper than treatment with ATG. If patients are hospitalized during the treatment period (usually 10 – 14 days with ATG as compared with 2 – 4 days with alemtuzumab), the difference in costs could even be larger.

Table 3. Comparison between ATG and alemtuzumab

	ATG	Alemtuzumab
Type of antibody	Polyclonal	Monoclonal
Targeted antigens	Multiple, including CD3, CD4, CD8, and CD40L	CD52
Targeted cells	Mainly T cells, to a lesser extent B cells and NK cells	T cells, B cells, NK cells, monocytes, macrophages, dendritic cells, eosinophils, mast cells
Route of administration	Intravenous, mostly via central venous catheter	Subcutaneous and intravenous
Dosing regimen	Based on bodyweight and lymphocyte / thrombocyte counts	Fixed dose
Treatment duration	Usually daily dosing for 10 – 14 days	Single dose, or two doses on two consecutive days
Side effects during or shortly after infusion	Fever, chills, dyspnea, nausea, diarrhea, headache, general pain, and pulmonary edema	Generally none when given subcutaneously
Costs of treatment (drugs only)	Variable, ranging from €850 - €3800	Fixed, €1000

Conclusion

Given the central role of T cells in the alloimmune response, anti-T cell antibodies like ATG and alemtuzumab are rational tools in the treatment of renal allograft rejection. Due to a more favorable side effect profile and lower costs, high-dose steroids remain the primary therapy for a first rejection episode. Severe or steroid-resistant renal allograft rejections are commonly treated with a 10- to 14-day course of ATG. This treatment is very effective, but is also associated with frequent infusion reactions and an increased incidence of infections and PTLD. Moreover, repeated use of ATG can be hampered by the development of antibodies against rabbit immunoglobulins. Alemtuzumab is commonly administered as an induction agent in the prophylaxis of rejection, but data on its use for treatment of acute rejection are limited. Administration is easy and nearly

devoid of side effects, but the long-term efficacy and safety as antirejection treatment are currently difficult to judge. There is a demand for other highly effective anti-T cell antibodies that lack the side effects of ATG and alemtuzumab.

Chapter 3

Use of monoclonal antibodies in renal transplantation

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Abstract

Monoclonal antibodies are applied in various settings in renal transplantation. Depleting T cell antibodies are used for treatment of steroid-resistant acute rejection and as induction therapy to reduce the intensity of concomitant immunosuppressive drug therapy. Induction therapy with the nondepleting IL-2 receptor antagonists basiliximab and daclizumab, added to cyclosporine-based regimens, reduces the incidence of acute rejection without side effects. However, an increase in long-term graft and patient survival has not been demonstrated yet. The B cell-targeting antibody rituximab is used in blood group ABO-incompatible transplantation, in desensitization protocols, and for treatment of antibody-mediated rejection. Eculizumab interrupts the complement pathway and is a promising tool for the treatment of antibody-mediated rejection and posttransplant hemolytic–uremic syndrome.

Introduction

For patients with end-stage renal disease, renal transplantation is the treatment of choice to improve quality of life and increase life expectancy. Next to the surgical challenges of this procedure, the manipulation of the alloimmune response is crucial for a successful renal transplantation. The alloimmune response consists of a recognition phase and an effector phase, in which different cells and molecules are involved, as described in chapter 2. While alloantibodies can be detrimental for the renal allograft, the general efficacy of antibodies to bind their target has fuelled the interest in the use of antibodies for therapeutic purposes. Because immunoglobulins cannot pass the cell membrane, all targets are either found on the cell surface or are noncellular targets (e.g., complement factors). Given the central role that T cells play in the alloimmune response, it is not surprising that most efforts to control this response with antibodies have been directed toward the development of various kinds of anti-T cell antibodies. More recently, antibodies against B cells and soluble molecules have gained interest for use in transplantation.

In the next paragraph, we will start with a brief overview of the use of antibodies in renal transplantation. Subsequently, the various monoclonal antibodies that are used in renal transplantation are described in more detail (see **Table 1** for an overview of currently used monoclonal antibodies).

Intervention in the alloimmune response with antibodies

In 1899, Metchnikoff was the first to use rabbit immune serum to remove leukocytes from human blood. More than 60 years later, the first use of rabbit antiserum to prevent allograft rejection in renal transplant recipients was reported. Nowadays, polyclonal rabbit antithymocyte globulin (ATG; Genzyme, Cambridge, MA, USA) is still commonly used to prevent and treat allograft rejection.

Where polyclonal antibodies target a broad range of molecules, monoclonal antibodies have a single molecular target. Monoclonal antibody production starts with fusing B cells from immunized hosts with immortalized myeloma cells, thereby creating so-called hybridomas. After selecting the hybridomas that produce the desired antibody, these hybridomas are cloned and the monoclonal antibody is harvested from the supernatant. Usage of monoclonal antibodies provides the ability to specifically target cells or

molecules involved in the alloimmune response. A rational strategy to achieve selective immunosuppression would be to target antigens that are expressed on lymphocytes that respond to alloantigens, but are absent on resting lymphocytes. The classical example of such a target is the -chain of the receptor for IL-2 (CD25) [64]. As outlined later, anti-CD25 antibodies have an important place in current treatment regimens after renal transplantation. While the prototypes of monoclonal antibodies were mostly of mouse origin and elicited human antimouse immunoglobulin antibodies, the currently used chimeric and humanized antibodies are far less immunogenic **(Box 1)**.

Box 1. Naming of monoclonal antibodies.

The naming of (new) monoclonal antibodies follows a strict pattern that has been described by the WHO in the International Nonproprietary Names (INN) Working Group Meeting on Nomenclature for Monoclonal Antibodies. The names of monoclonal antibodies are composed of a prefix, a substem A, a substem B and a suffix. The common suffix for monoclonal antibodies is 'mab'. Substem B indicates the species on which the immunoglobulin sequence of the monoclonal antibody is based. For example, 'a' for rat, 'xi' for chimeric, 'zu' for humanized and 'u' for human. Substem A indicates the target class (molecule, cell or organ), for example, 'c(i)' for cardiovascular, 't(u)' for tumor, and 'l(i)' for immunomodulating. Finally, the prefix can be random to contribute to a distinctive name. Therefore, basi-li-xi-mab, is a monoclonal antibody of chimeric origin, with an immunomodulating target. The only monoclonal antibody that does not comply with this naming is muromonab-CD3, which is short for murine monoclonal antibody against CD3.

Anti-T cell monoclonal antibodies are especially used for treatment of acute rejection and as induction therapy during the first days or weeks after transplantation. Induction therapy can have several goals that are not mutually exclusive: to prevent acute rejection, to induce tolerance, to minimize steroid use, or to postpone or reduce the treatment with calcineurin inhibitors, especially in cases where the risk of acute tubular necrosis is high. The anti-B cell monoclonal antibody rituximab is used in ABO-incompatible transplantation, in desensitization protocols, and for treatment of antibody-mediated rejection. Moreover, it has an important place in the treatment of posttransplant lymphoproliferative disease. Currently, no monoclonal antibody is used or registered for maintenance immunosuppression.

Muromonab-CD3 (OKT3): mouse anti-CD3

The first monoclonal antibody approved for use in human renal transplantation was muromonab-CD3 (Orthoclone OKT3, Janssen- Cilag, Beerse, Belgium). It targets the CD3 protein on the surface of T cells, which is part of the T cell receptor complex. Immediately after administration, CD3+ T cells are cleared from the circulation. In a prospective, randomized, multicenter trial, the efficacy in reversing acute rejection of renal transplants from deceased donors was clearly established. Not only did full-course treatment with OKT3 lead to superior reversal rates of acute rejection compared with high-dose steroids (94 vs. 75%), it also showed efficacy in treating steroid-resistant rejections [65]. OKT3 has also been used as induction therapy, although this is not based on randomized trials, and the drug has never been formally approved for this indication.

Unfortunately, OKT3 has numerous adverse effects. Especially the first administration can be followed by a cytokine release syndrome, which is caused by a transient activation of T cells, before they undergo cell lysis [37]. The clinical manifestations of this syndrome are fever, chills, headache, dyspnea, myalgia and hypotension. The release of cytokines probably also contributes to diarrhea, pulmonary edema, intra-allograft thrombosis, aseptic meningitis and temporary hearing loss. Furthermore, patients given OKT3 are more likely to develop serious infections (e.g., cytomegalovirus, Epstein–Barr virus, *Listeria* and *Mycobacterium*), and posttransplant lymphoproliferative disease [52]. For the treatment of acute rejection, OKT3 is outperformed by ATG, which is at least as effective and has a somewhat more favorable side effect profile [11]. The declining usage of OKT3 has recently led to its removal from the market.

Alemtuzumab: humanized (rat) anti-CD52

CD52 is expressed on T cells, B cells, NK cells, monocytes, macrophages and dendritic cells. Early clinical trials with rat depleting anti-CD52 antibodies (Campath-1G and Campath-1M) as induction therapy in renal transplantation showed effective lymphocyte depletion with reduced incidence of acute rejection, but with immunogenicity and also an increased incidence of infections [66]. The immunogenicity was reduced, but not completely eliminated, by humanizing the antibody (alemtuzumab; Campath-1H, Genzyme). The depletion of T cells caused by alemtuzumab is long lasting; T cell levels recover to only 50% of baseline at 36 months after administration [22].

Alemtuzumab is indicated for the treatment of chronic lymphatic leukemia. Experience with the off-label use of alemtuzumab for treatment of acute rejection after renal transplantation is scarce and no randomized trials have been published on this subject. Most articles report on a few patients, merely demonstrating that allograft rejection can be reversed with alemtuzumab [34, 35]. There is one larger trial describing 40 patients, in whom alemtuzumab was given because of steroid-resistant rejections, or rejections equal to or more severe than Banff 1B. Allograft survival was 74% after 1 year, with a patient survival of 95%. A total of 14 patients (35%) had infectious complications, of whom two patients (5%) died [31]. In another trial, 15 patients were treated with alemtuzumab for acute biopsy-proven rejection and compared with a historical control group of patients treated with methylprednisolone. All rejection episodes were reversed by alemtuzumab and 10-year allograft function was similar in both groups [30]. The use of alemtuzumab for the treatment of acute rejection may expand after the removal of OKT3 from the market.

Most experience with alemtuzumab stems from the use as induction therapy, especially in the setting of a reduced intensity of maintenance immunosuppression. In such a scenario, alemtuzumab might contribute to the induction of antigen-specific tolerance [67]. The use of alemtuzumab as add-on to standard triple or dual immunosuppression is considered to result in overimmunosuppression. In an initial trial with alemtuzumab induction therapy in 31 renal transplant recipients, a single dose of 30 mg was combined with low-dose cyclosporine maintenance immunosuppression. The incidence of early acute rejection episodes was approximately 20% and after 5 years of follow-up, there was no difference in incidence of rejection episodes, allograft function or allograft and patient survival, compared with patients who received cyclosporine, azathioprin and prednisolone [68]. Importantly, there were no differences in the incidence of malignancies or infections. Rejections in the alemtuzumab group tended to occur beyond the first year after transplantation. Likewise, alemtuzumab induction followed by tacrolimus monotherapy resulted in similar graft and patient survival as tacrolimus in combination with mycophenolate mofetil and steroids [50]. In this trial, alemtuzumab-treated patients showed less acute rejections but an increased incidence of CMV infections. Multiple studies, mostly retrospective or observational in nature, have confirmed these results and underline the ability of induction therapy with alemtuzumab to reduce the intensity of maintenance immunosuppression [55, 69]. However, in a pilot trial of 29 patients, the use of alemtuzumab with sirolimus monotherapy was associated with an increased risk of acute (humoral) rejection [70].

Table 1. Monoclonal antibodies that are currently used in renal transplantation.

Name	Area of application	Origin	Target and mechanism of action	Dosing regimen	Main side effects
Alemtuzumab	Treatment of acute rejection Induction therapy	Rat, humanized	CD52 on mononuclear cells, depleting antibody	Single or double dose of 20–30 mg each iv. or sc. Pediatric dosing unknown	First-dose reaction after iv. administration, allergic reaction and increased incidence of infections
Basiliximab	Induction therapy	Mouse, chimeric	CD25 on T cells, nondepleting antibody	Two doses of 20 mg iv., 4 days apart. Pediatric dosing two doses of 10 mg	None
Rituximab	Blood group ABO-incompatible transplantation Desensitization Treatment of acute rejection Induction therapy	Mouse, chimeric	CD20 on B cells, depleting antibody	Single dose of 375 mg/m^2 or two doses of 1000 mg, 2 weeks apart. Pediatric dosing 375 mg/m^2	Mild cytokine release syndrome, allergic reaction, neutropenia
Eculizumab	Treatment and prevention of antibody-mediated acute rejection Treatment of posttransplant hemolytic uremic syndrome	Mouse, humanized	Complement C5, blocks formation of membrane attack complex	Variable; loading dose of 1200 mg and weekly doses of 900 mg. Pediatric dosing unknown, Phase II trial underway	Insufficient data; increased risk of infection with encapsulated bacteria

iv.: Intravenous; sc.: Subcutaneous.

In several trials, alemtuzumab has been directly compared with other agents used for induction therapy, such as ATG or anti-CD25 antibodies. In general, the use of alemtuzumab was as least as effective as other induction agents in the prevention of acute rejection, while the number of drugs for maintenance immunosuppression could be reduced [71-74]. Although alemtuzumab causes profound depletion of T and B cells, the incidence of acute side effects is comparable with placebo. Commonly reported is an increased incidence of infections, although this is mainly observed in patients with a high overall burden of immunosuppression, either after transplantation or pretransplantation for the underlying renal disease.

Basiliximab: chimeric human–mouse anti-CD25

The first monoclonal antibodies targeting CD25 in humans were of mouse origin. Although effective in preventing acute rejection, the clinical applicability was limited by the development of antimouse immunoglobulin antibodies [75]. Basiliximab (Novartis Pharma, Basel, Switzerland) is a chimeric (human and mouse) nondepleting anti-IL-2 receptor antibody. In the first randomized, double-blind trial reported, 193 patients who received basiliximab were compared with 187 patients who received placebo [76]. Both groups received dual immunosuppressive therapy with cyclosporine and steroids. The incidence of biopsy confirmed acute rejection within 6 months after transplantation was 30% in the basiliximab group, compared with 44% in the placebo group. The incidence of steroid-resistant first rejection episodes that required anti-T cell antibody therapy was significantly lower in the basiliximab group (10 vs. 23%). However, the incidence of graft loss at 12 months posttransplantation was similar in both groups (12 vs. 13%). Comparable results were found in another randomized, controlled trial 2 years later [77].

Patients treated with a triple immunosuppressive regimen, either azathioprin or mycophenolate mofetil added to cyclosporine and steroids, also benefit from induction therapy with basiliximab [78]. Basiliximab is generally associated with a tolerability profile that is similar to that reported with placebo.

Daclizumab: humanized (mouse) anti-CD25

Daclizumab (Roche, Basel, Switzerland) is a humanized anti-IL-2 receptor antibody. The efficacy of daclizumab in preventing acute rejection after renal transplantation was analyzed in two multicenter, randomized, placebo-controlled trials, in which maintenance immunosuppression consisted of triple and dual therapy, respectively. In both studies, a significant decrease in the incidence of biopsy confirmed acute rejection was observed, without an increase in side effects [79, 80].

Similarities & differences between basiliximab & daclizumab

In 2003, a meta-analysis of trials comparing basiliximab or daclizumab with placebo as induction therapy confirmed that the incidence of biopsy-proven acute rejection at 6 months is reduced by approximately 50% (95% CI: 37–58%) with a similar effect size for basiliximab and daclizumab [81]. All studies included in this meta-analysis used a cyclosporine-based maintenance regimen. By now, most immunosuppressive regimens are based on tacrolimus, which appears to be a somewhat stronger immunosuppressant than cyclosporine [82]. The number of clinical trials studying the effects of IL-2 receptor antagonists when added to a tacrolimus-based regimen is very limited and results from these studies are conflicting. In one trial, the addition of a single dose of daclizumab to tacrolimus, mycophenolate mofetil and steroids, seemed to reduce the incidence of acute biopsy-proven rejection during the first 6 months after transplantation compared with placebo (6 vs. 16%) [83]. In pediatric recipients, the addition of basiliximab to a tacrolimus-based regimen had no benefits [84]. In a recent meta-analysis of the effects of IL-2 receptor antagonists by the Cochrane Renal Group, three tacrolimus-based and 26 cyclosporine-based regimens were included. Although combined analysis still confirmed the reduction of the incidence of acute rejection within 1 year after transplantation compared with placebo (relative risk: 0.72; 95% CI: 0.64–0.81), the effect was not significant in the subanalysis of the three tacrolimus-based studies (relative risk: 0.66; 95% CI: 0.28–1.57) [85].

Next to the reduced incidence of acute rejection within 1 year after transplantation, this meta-analysis showed significant reductions in graft loss, CMV disease, early malignancies and transplant dysfunction, although these differences were not sustained more than 1 year after transplantation.

Studies with IL-2 receptor antibodies have mostly been performed in low-immunological-risk patients, and some studies report that in high-risks patients, ATG might be superior to IL-2 receptor antibodies for the prophylaxis of acute rejection [86-88], although the Cochrane meta-analysis does not support this conclusion. In immunologically low-risk patients, the use of IL-2 receptor antagonists enables the avoidance of steroids in patients treated with tacrolimus and mycophenolate mofetil [89, 90].

In theory, targeting of the IL-2 receptor could also affect regulatory T cells with a CD4+CD25+FOXP3+ phenotype, which might impede graft acceptance. Although a shift of regulatory T cells from the CD25+ to the CD25- compartment during treatment with IL-2 receptor antagonists has been observed, several studies have demonstrated that the suppressive function of the regulatory T cells was maintained [91-93].

Based on the data summarized above, induction therapy with IL-2 receptor antagonists is recommended in the 2009 Kidney Disease: Improving Global Outcome (KDIGO) clinical practice guideline for the care of kidney transplant recipients [29]. The last published survey of the Organ Procurement and Transplantation Network (OPTN) in the USA reported that in 2006 approximately 28% of renal transplantations were performed with IL-2 receptor antagonist induction therapy [94]. It is likely that the KDIGO recommendations will cause an increase in this percentage in the future.

The only differences between basiliximab and daclizumab are in dosing and length of therapy. In clinical trials, up to 20% of patients did not receive the scheduled five doses of daclizumab, but it has been shown that two doses of daclizumab can provide prolonged blockade of the IL-2 receptor [95]. The similar efficacy of basiliximab and daclizumab, combined with the lack of approval for a limited dosing regimen of daclizumab, probably contributed to the voluntary withdrawal of daclizumab from the market in 2009.

Rituximab: chimeric human–mouse anti-CD20

Rituximab (MabThera, Hoffmann-La Roche, Basel, Switzerland) is a chimeric human–mouse antibody against the CD20 molecule on B cells. After binding, it leads to clearance of B cells from the circulation. Rituximab is approved for the treatment of B cell non-Hodgkin's lymphomas and severe rheumatoid arthritis.

In renal transplantation, the off-label use of rituximab is part of the recipient treatment when an ABO-incompatible living donor donates a kidney. These transplantations were previously only performed with intensive immunosuppression, plasmapheresis to remove anti-A and/or anti-B antibodies, and splenectomy. In 2001, the group of Tydén in Sweden replaced plasmapheresis with specific immune absorption of anti-A and/or anti-B antibodies, and splenectomy with a single dose of rituximab [96]. The initial experience with this regimen in 15 patients showed an incidence of acute rejection after 2 years of only 6.7%. Patient and graft survival were 100 and 86%, respectively. Moreover, cytokine release syndrome was not described and the incidence of infections or malignancies was not increased [97]. The favorable results of similar protocols have been confirmed by others, and have led to a substantial rise in the number of ABO-incompatible living donor renal transplantations [98].

Patients with a high level of anti-HLA antibodies or with a positive cross-match wait long, and sometimes in vain, for a kidney. To reduce the level of anti-HLA antibodies, 76 selected patients received rituximab and intravenous immunoglobulin before transplantation [99, 100]. Although the incidence of acute rejection after transplantation was relatively high (37%), the biggest potential gain of this approach is the possibility to transplant patients, who otherwise would have continued dialysis treatment.

In steroid-resistant rejections, clusters of B cells can be found in the renal interstitium, which are possibly associated with worse graft survival [3, 101]. Although most reports involve selected patient groups, acute or chronic antibody-mediated rejection or steroid-resistant rejection episodes can respond to rituximab (even when there was no response to ATG and/or plasmapheresis) with clusters of B cells successfully being removed from the interstitium [102-105].

The data on the use of rituximab as induction therapy are limited. Based on their favorable experience with rituximab in ABO-incompatible transplantations, Tydén et al. evaluated the effectiveness and safety of single-dose rituximab induction therapy compared with placebo, added to tacrolimus-based triple therapy. The incidence of acute rejections after 6 months was 12% in the rituximab group and 18% in the placebo group, which was not significantly different [106]. Another trial, using two doses of rituximab with steroid-free maintenance immunosuppression, was halted early due to an excess rate of acute cellular rejection. This raises the possibility that regulatory B cells may be depleted by rituximab [107].

Rituximab has also successfully been used in the treatment of recurrent focal segmental glomerulosclerosis and membranous nephropathy after renal transplantation. However, controlled studies for these indications are lacking and in many of the reported cases the efficacy of rituximab is difficult to assess since it was combined with other immunosuppressive drugs or with plasmapheresis [108]. Finally, rituximab has an important place in the treatment of posttransplant lymphoproliferative disorder [109].

Administration of rituximab can be followed by a mild cytokine release syndrome. Although rituximab induces long-lasting B cell depletion, the risk of infections appears to be limited [97, 110, 111]. Several cases of late-onset neutropenia have been described [112].

Eculizumab: humanized (mouse) anti-C5

Eculizumab (Alexion, Cheshire, CT, USA) is a humanized (human–mouse) monoclonal antibody directed against the complement protein C5. It blocks the cleavage of C5 and halts the formation of the membrane attack complex. It is successfully used in paroxysmal nocturnal hemoglobinuria [113]. Eculizumab could potentially be beneficial in renal transplantation for treatment or prevention of antibody-mediated rejection, which is accompanied by activation of the complement system. The first case reports show that eculizumab can be safely and effectively used in selected patients to prevent injury from CDC [114]. This creates a window of opportunity for other therapies to clear the donor-specific antibodies. After renal transplantation, hemolytic uremic syndrome (HUS) may occur either as a recurrent or de novo form. During recent years, it has become clear that dysregulation of the complement alternative pathway leading to continuous complement activation is involved in the pathogenesis of HUS [115]. Several case reports have now demonstrated that treatment with eculizumab can improve outcome in patients with HUS after renal transplantation [116, 117]. Since eculizumab diminishes the defense against encapsulated bacteria, especially meningococci, patients should ideally undergo meningococcal vaccination prior to receiving the first eculizumab treatment. The response to this vaccination could however be suboptimal, especially during (intensified) immunosuppressive therapy.

Chapter 4

Effect of a single intraoperative high-dose of ATG-Fresenius on delayed graft function in donation after circulatory-death donor renal allograft recipients: a randomized study

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Abstract

Objectives: Reducing the incidence of delayed graft function after transplant with donation after circulatory death donor renal allografts would facilitate managing recipients during their first weeks after a transplant. To reduce this incidence, in most studies, induction therapy with depleting anti-T-lymphocyte antibodies is coupled with a reduction of the dosage of the calcineurin inhibitor. The separate effect of anti-T cell therapy on the incidence and duration of delayed graft function is therefore difficult to assess.

Patients and Methods: We performed a randomized study to evaluate the effect of a single intraoperative high-dose of anti-T-lymphocyte immunoglobulin (ATG)-Fresenius (9 mg/kg body weight) on the incidence of delayed graft function. Eligible adult recipients of a first donation after circulatory death donor renal allograft were randomly assigned to ATG-Fresenius or no induction therapy. Maintenance immunosuppression consisted of tacrolimus, in an unadjusted dose, mycophenolate mofetil, and steroids.

Results: The study was prematurely terminated because of a lower-than-anticipated inclusion rate. Baseline characteristics were comparable in the ATG-Fresenius group (n = 28) and the control group (n = 24). Twenty-two patients in the ATG-Fresenius group (79%) had delayed graft function, compared with 13 in the control group (54%; p = 0.06). Allograft and patient survival were comparable in both groups. Serious adverse events occurred more frequently in the ATG-Fresenius group than they did in the control group (57% vs. 29%; $p < 0.05$).

Conclusions: Intraoperative administration of a single high-dose of ATG-Fresenius in donation after circulatory death donor renal allograft recipients, followed by triple immunosuppression with an unadjusted tacrolimus dose, seems ineffective to reduce the incidence of delayed graft function. Moreover, this was associated with a higher rate of serious adverse events (EudraCT-number, 2007-000210-36.)

Introduction

The increased waiting list for renal transplant has prompted the use of so-called "expanded criteria donors" to increase the number of renal allografts available for transplant. Donation after circulatory death (DCD) has emerged as a satisfactory option to provide renal allografts with patient and graft survival rates similar to those obtained with renal allografts from donation after brain death donors [118]. A major problem with transplant of renal allografts from DCD donors is the high incidence of delayed graft function (around 40% to 50%, but might be as high as 80%) [119]. This frequently results in the continued need for dialysis for some time after the transplant, with associated increases in morbidity and mortality, and prolonged hospital stay [120]. Moreover, the lack of graft function requires performing graft biopsies at regular intervals to exclude acute rejection. Finally, delayed graft function is a risk factor for acute rejection and graft loss, although the detrimental effect of delayed graft function on graft survival appears to be much weaker in transplants with DCD donor renal allograft than in transplants with donation after brain death donor renal allografts [121, 122]. Consequently, reducing the incidence of delayed graft function after transplant with DCD donor renal allografts would facilitate managing recipients during the first weeks after a transplant and potentially improve long-term outcomes.

Renal allografts from a DCD donor have prolonged warm ischemia periods and therefore, have more severe ischemia-reperfusion–associated tissue damage. Ischemia-reperfusion injury involves a cascade of deleterious steps, including increased cytokine synthesis and leukocyte-mediated tissue damage. Next to neutrophils, T cells have been identified as important cellular mediators in ischemia-reperfusion injury [123]. T cell depletion at the time of transplant may reduce the extent of tissue damage after ischemia-reperfusion injury [120].

Various depleting anti-T-lymphocyte antibodies are available for use in transplant. They include rabbit anti-human thymocyte immunoglobulin (Thymoglobulin, Genzyme), equine anti-thymocyte immunoglobulin (Atgam, Pfizer), and rabbit anti-human activated T-lymphocyte immunoglobulin (ATG-F; ATG-Fresenius, Fresenius Biotech GmbH). Rabbit anti-human activated T-lymphocyte immunoglobulin consists of highly purified immunoglobulins, derived from rabbits after immunization with a T-lymphoblast cell line (i.e., Jurkat cell line). Administering the polyclonal anti-T-lymphocyte antibody ATG-F results in rapid T cell depletion. Rabbit anti-human activated T-lymphocyte immunoglobulin also has some effects on other cells of the

immune system, namely proliferating B-lymphocytes and other antigen presenting cells [16, 124].

In previous clinical studies on induction therapy with depleting anti-T-lymphocyte antibodies, this treatment was usually coupled to a reduction of the dosage of the calcineurin inhibitor (either tacrolimus or cyclosporine). Because calcineurin inhibitors can retard the recovery of graft function after renal transplant, the separate effect of anti-T cell therapy on the incidence and duration of delayed graft function is difficult to judge in these studies [125]. Therefore, in our study, a different study protocol was chosen. A regular, unadjusted dose of tacrolimus was used in both the ATG-F group and control group to evaluate the effect of ATG-F on ischemia-reperfusion injury.

In this study, we evaluate whether a single, intraoperative high-dose of ATG-F added to a triple immunosuppressive drug regimen with an unadjusted dose of tacrolimus, could reduce the incidence and duration of delayed graft function after transplant with a DCD donor renal allograft.

Patients and Methods

This multicenter, randomized, open label study was conducted in 4 university centers in The Netherlands. The study was conducted in compliance with the applicable regulatory requirements and the Declaration of Helsinki. Written, informed consent was obtained from all patients. During the study, no changes were made to the design of the study. The conduct of the study was continually monitored by independent study nurses.

All patients (aged 18 years) who were candidates to receive a renal allograft from a DCD donor were eligible for this study. Acceptability criteria for donor age, and warm and cold ischemia times were according to local protocols. Exclusion criteria were a previous transplant or proposed transplant with multiple organs (e.g., kidney-pancreas transplant); blood group incompatibility; current pregnancy or history of more than 3 pregnancies; lack of consistent data on a panel reactive antibody; known presence of antibodies against rabbit immunoglobulin or previous treatment with rabbit immunoglobulin; known intolerance to any component of basal immunosuppression; HIV-positivity; leukocytes < 3.0 10^9/L and/or platelets < 50 10^9/L before transplant; (cured) malignancy (with the exception of basocellular or spinocellular skin cancer); and pulmonary edema or other signs of overhydration. Patients were randomly assigned (1:1) to either the ATG-F group or the control group. Treatment assignments were

randomized at the coordinating center using a computer-derived algorithm. Treatment assignments were printed on paper and put in concealed, numbered envelopes. Participants were stratified for the age of the recipient (< 50 and 50 years) and the length of the first warm ischemia time (< 30 and 30 minutes). Patients were assigned a consecutive number by the participating center, in the order in which they entered the study. The consecutive number corresponded with the envelope containing the assigned treatment, which was opened after eligibility of the patient was finally established and the patient was ready for treatment.

Patients in the ATG-F group received a single high-dose of ATG-F IV (9 mg/kg body weight, diluted in 500 mL saline) intraoperatively. Before the infusion of ATG-F, patients received 250 mg of methylprednisolone IV. The infusion of ATG-F was given in 4 hours and did not need to be completed before reperfusion of the graft. Afterward, patients received triple immunosuppression with tacrolimus, mycophenolate mofetil, and steroids. In the control group, patients received only 250 mg of methylprednisolone intraoperatively and equal triple immunosuppression after transplant.

In both groups, patients were treated with a regular, unadjusted dose of tacrolimus, to enable evaluation of the separate effect of adding ATG-F. The initial dosage of tacrolimus was 0.2 mg/kg/day orally, starting within 24 hours posttransplant. The tacrolimus dosage was adjusted to a target trough level of 15 to 20 mg/L in the first 2 weeks posttransplant, 10 to 15 mg/L during the 3 to 6 weeks after transplant, and 5 to 10 mg/L thereafter. Mycophenolate mofetil was started at a dosage of 2000 mg/day. After 2 weeks, the dosage was decreased to 1500 mg/day, unless the body weight was more than 90 kg. In patients with delayed graft function, the starting dosage was 1500 mg/day to reduce adverse events caused by an accumulation of metabolites. Prednisone was given in a dosage of 100 mg IV for the first 3 days after the operation. Afterward, the dosage of prednisone was tapered according to local practices. For prophylaxis of Pneumocystis jiroveci pneumonia, trimethoprim/sulfamethoxazole was given in a dosage of 480 mg daily. For prophylaxis of cytomegalovirus disease, valganciclovir was given in case of a seropositive donor and seronegative recipient in a dosage adjusted to allograft function.

The primary endpoint of this study was the incidence of delayed graft function, defined as the need for dialysis following transplant. Secondary endpoints were the duration of initial delayed graft function (defined as the interval between the day of transplant and the last day of dialysis), and incidence of primary non-function of the allograft. Moreover,

at 3 months after transplant, the incidence of acute rejection (clinically treated and biopsy-proven), allograft function and proteinuria, patient and graft survival, incidence of arterial hypertension, use of antihypertensive drugs, incidence of hyperlipidemia, and incidence of posttransplant diabetes mellitus were recorded. As safety parameters, the incidence of infections, especially cytomegalovirus, the incidence of malignancies during the first 3 months after transplant, and the incidence of serious adverse events were recorded. Serious adverse events were defined as any untoward medical occurrence that at any dose resulted in death, was life-threatening, required inpatient hospitalization or prolongation of existing hospitalization, or was otherwise medically significant to prevent or reduce permanent impairment or damage. Primary and secondary endpoints were not changed during the study.

At any time during the application of ATG-F or shortly after the infusion, there is a risk of anaphylactic reactions including a drop in blood pressure, chest pain, fever, or urticaria. Because the study used a single dose of ATG-F, the only reason for discontinuation of the treatment was clinically significant symptoms during infusion. Therapy was not discontinued if the symptoms remained mild and reversible.

Sample size determination was made under the assumption that the rate of delayed graft function would be 80% in the control group, with a reduction to 60% or less with ATG-F. A sample size of 80 patients per group provided at least 80% power, with
= 2.5% one-sided, to detect this difference. Taking possible dropouts into account, the study was planned with 90 patients per group, requiring 180 patients in total. No interim analysis was planned nor performed in this study.

We performed overall group comparisons using a chi-square test or Fisher exact test (if counts per group were below 5). For continuous variables, we used either an unpaired T test (normally distributed data) or a Wilcoxon Mann-Whitney U test (not-normally distributed data). All statistical tests were two-sided, and $p < 0.05$ was considered statistically significant. Patients who underwent transplantation were evaluated in an intention-to-treat analysis. Because the number of patients was small, the primary and secondary endpoints were not analyzed within strata.

Results

The study was prematurely terminated in June 2010 because of a much lower-than-anticipated inclusion rate, without the prospect of improvement. Between January 2008 and June 2010, all adult patients (n = 151) who were candidates to undergo transplant with a DCD donor renal allograft were assessed for eligibility. In total, 54 patients could be randomized (**Figure 1**). Most patients were ineligible because they met exclusion criteria, for example, a previous transplant. In addition, many patients could not be included because the preparation time for the transplant was too short to obtain proper informed consent. The 54 patients who were included for randomization did not differ from the 97 patients not included with respect to recipient age, sex, cause of end-stage renal disease, and ischemia times (data not shown).

Of the 54 included patients, 30 were randomized for treatment with ATG-F. The data of two patients in the ATG-F group were not analyzed because the transplant was cancelled because of the bad quality of the allograft and a positive crossmatch, respectively. Although one patient in the ATG-F group did inadvertently not receive ATG-F, this patient was included in the analysis. If this patient were excluded in a per-protocol analysis, the outcome on all endpoints did not change. All randomized patients finished the 3-month follow-up. Patients within strata were equally randomized between ATG-F and control treatment. The groups also showed no significant differences with respect to donor and recipient characteristics (**Table 1**).

The incidence of delayed graft function did not significantly differ between both groups (79% in the ATG-F group vs. 54% in the control group; $p = 0.06$; **Table 2**). Four patients in the ATG-F group and two in the control group required only one dialysis session after transplant. The duration of delayed graft function, the incidence of primary non-function, and the incidence of biopsy-proven rejection did not differ between the ATG-F and the control group. At 3 months after transplant, patient and graft survival were 100% and 96% in the ATG-F group versus 96% and 83% in the control group. Serum creatinine was not different between groups at any moment after transplant.

One day after transplant, the absolute lymphocyte count was lower in the ATG-F group as compared to the control group (0.18 10^9/L, range, 0.0-0.48 10^9/L vs. 0.59 10^9/L, range 0.0-1.6 10^9/L; $p < 0.01$). Two weeks after transplant, this difference between the ATG-F and control group disappeared. The thrombocyte count one day after transplant also was lower in the ATG-F group (115 10^9/L, range 56-256 10^9/L) as compared to the control group (191 10^9/L, range, 81-336 10^9/L; $p < 0.01$). This difference was no longer

present 3 weeks after transplant. Three patients (11%, 3/28) had a severe reaction when given ATG-F, mainly hypotension. Moreover, an additional two patients in the ATG-F group had signs of hemolysis the day after transplant, for which no other explanation than administering ATG-F was available. Serum sickness was not reported, although one patient in the ATG-F group was found to have a positive titer of anti-rabbit immunoglobulin antibodies (80 U/L) at the time of transplant (without known exposure to rabbit proteins or previous known positive anti-rabbit immunoglobulin antibodies).

Figure 1. Enrolment of patients in the study

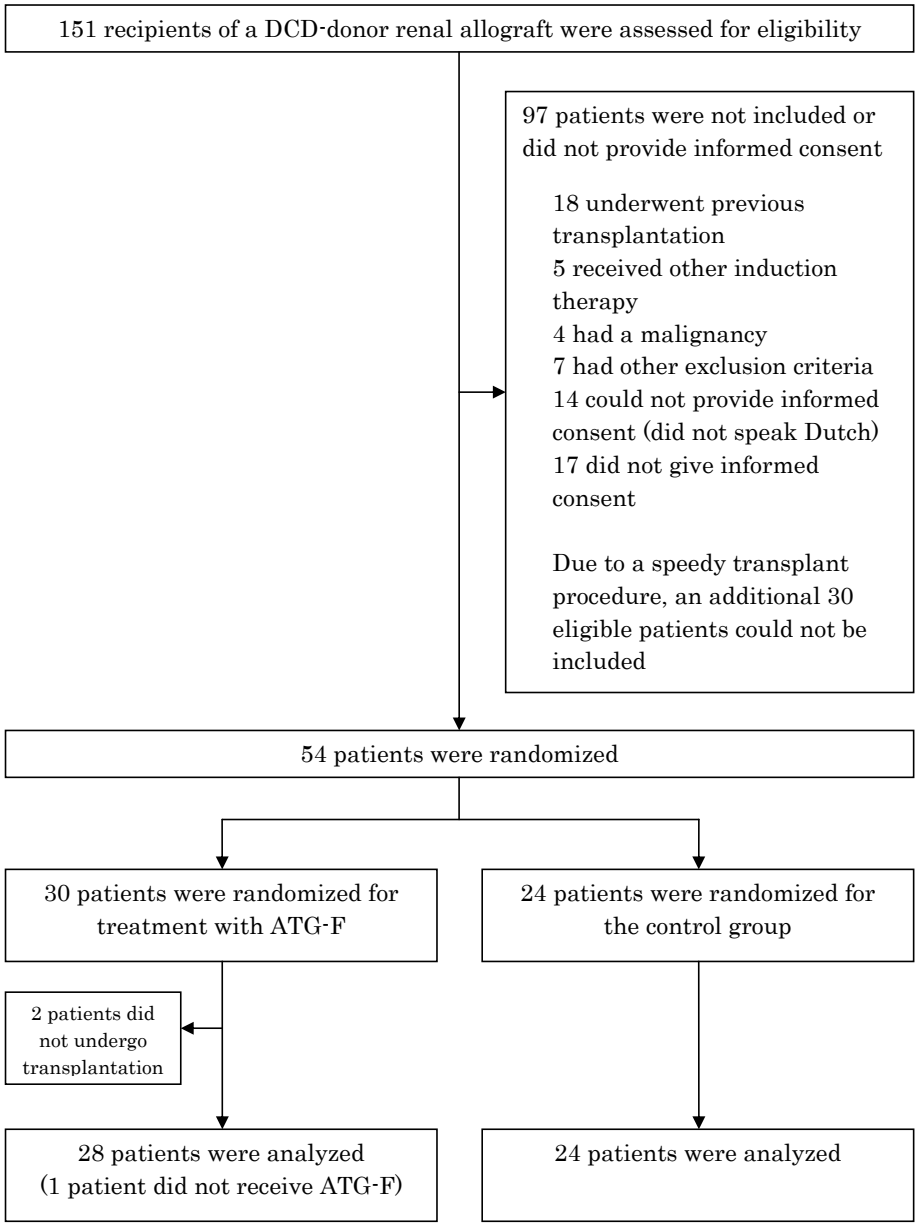

Table 1. Baseline characteristics of renal allograft recipients.*

	ATG-F group (n = 28)	Control group (n = 24)
Mean age, years (range)	54 (21-70)	56 (24-68)
Sex – no. (%)		
Male	18 (64)	17 (71)
Female	10 (36)	7 (29)
Cause of end-stage renal disease, no. (%)		
Glomerulonephritis	9 (32)	5 (21)
Polycystic kidney disease	6 (21)	4 (17)
Diabetic nephropathy	0 (0)	2 (8)
Hypertension	2 (7)	3 (13)
Other	11 (39)	10 (42)
First warm ischemia time – min	18 ± 4	19 ± 6
Cold ischemia time – hr	16.4 ± 5.4	16.6 ± 4.5
Anastomosis time – min	33 ± 11	31 ± 11
Stratification – no. (%)		
Recipient age 50 years and first warm ischemia time <30 min	20 (71)	18 (75)
Recipient age <50 years and first warm ischemia time <30 min	8 (29)	4 (17)
Recipient age 50 years and first warm ischemia time 30 min	0 (0)	2 (8)
Recipient age <50 years and first warm ischemia time 30 min	0 (0)	0 (0)
Leukocyte count, 10^9/l	8.4 ± 2.5	7.8 ± 2.4
Absolute lymphocyte count, 10^9/l	1.7 ± 0.9	1.5 ± 0.7
Thrombocyte count, 10^9/l	242 ± 115	238 ± 73

* Values are given as means ± SD, unless stated otherwise. There were no statistically significant differences between groups.

Table 2. Primary and secondary endpoints at three months after renal transplantation.*

	ATG-F group (n = 28)	Control group (n = 24)	Absolute risk difference (95% CI)
Primary endpoint			
Incidence of delayed graft function, no (%)	22 (79)	13 (54)	25% (-1 to 48)
Secondary endpoints			
Duration of delayed graft function – days; (range)	10.1 (1-24)	16.4 (3-47)	-
Incidence of primary non-function, no. (%)	1 (4)	3 (13)	-9% (-28 to 7)
Incidence of treatment for rejection, no. (%)	6 (21)	7 (29)	-8% (-32 to 16)
Biopsy performed, no. (%)	12 (43)	11 (46)	-3% (-29 to 24)
Biopsy-proven rejection, no. (%)	2 (7)	2 (8)	-1% (-20 to 16)
Patient survival, no. (%)	28 (100)	23 (96)	4% (-8 to 20)
Graft survival, no. (%)	26 (96)	20 (83)	13% (-9 to 30)
Serum creatinine week 2, µmol/l (range)	567 (115-1020)	426 112-979)	-
Serum creatinine month 1, µmol/l (range)	289 (123-814)	247 (91-586)	-
Serum creatinine month 2, µmol/l (range)	191 (95-562)	238 (79-701)	-
Serum creatinine month 3, µmol/l (range)	178 (103-352)	180 (80-437)	-
Proteinuria month 1, g/l (range)	0.39 (0-1.94)	0.84 (0.1-5.2)	-
Proteinuria month 2, g/l (range)	0.24 (0-0.58)	0.34 (0-1.56)	-
Proteinuria month 3, g/l (range)	0.21 (0-0.47)	0.20 (0-0.54)	-
Incidence of hypertension, no (%)	23 (82%)	20 (83%)	-1% (-22 to 21)
Number of anti-hypertensive drugs (range)	1.7 (1-3)	1.6 (1-3)	-

Side effects			
Patients with at least one infection, no. (%)	17 (63)	9 (38)	25% (-2 to 50)
CMV infection, no. (%)	3 (11)	2 (8)	3% (-17 to 21)
Malignancies, no. (%)	0 (0)	0 (0)	0%
Posttransplant diabetes mellitus, no. (%)	12 (46)	4 (20)	26% (-2 to 50)

* Values are given as means. There were no statistically significant differences between groups.

Discussion

The main objective of this randomized multicenter study was to test the efficacy of ATG-F to reduce the incidence of delayed graft function after DCD donor renal transplantation. The premature termination of our study does not allow drawing firm conclusions related to our main objective. Nonetheless, our study indicates that the addition of a single intraoperative dose of ATG-F to standard triple immunosuppressive therapy, with an unadjusted tacrolimus dose, is not effective to reduce the incidence or duration of delayed graft function and might even be associated with a higher incidence of serious adverse events.

Interestingly, there was also no effect of ATG-F on the incidence of acute rejection. In other studies, induction therapy with a single dose of ATG-F or other anti-T-lymphocyte immunoglobulins universally reduced the incidence of acute rejection [126-131]. Because we noticed a profound lymphocytopenia and a mild thrombocytopenia in the ATG-F group, ineffectiveness of the ATG-F itself seems an unlikely explanation for the lack of a beneficial effect on the incidence of delayed graft function and incidence of rejection. Rather, it appears that either the contribution of T cells in the pathogenesis and recovery of acute tubular necrosis after transplant with a DCD donor renal allograft is limited, or that the positive effect of ATG-F is counterbalanced by the negative effect of other factors. The reaction that accompanied the infusion of ATG-F could be one of those factors. Five patients had hypotension, thrombocytopenia, or fever. Although no cytokines were measured, these symptoms are known to be caused by a release of cytokines [132]. This cytokine release syndrome could have contributed to a more proinflammatory environment, leading to more severe ischemia-reperfusion injury and worse outcomes. Hypotension, per se, also could have worsened ischemia-reperfusion injury. All patients with an infusion reaction developed delayed graft function, although

Table 3. Serious adverse events reported during the study.

	ATG-F group (n = 28)	**Control group (n = 24)**
Incidence of serious adverse events, no. of patients (%)*	16 (57)	7 (29)
Total number of reported serious adverse events (no.)	23	9
Severity of serious adverse events		
Death	0	1
Unsuccessful resuscitation after cardiac arrest at the fifth post-operative day	*0*	*1*
Life-threatening	2	0
Dissection of the thoracic and abdominal aorta on the third postoperative day	*1*	*0*
Intraoperative myocardial infarction	*1*	*0*
New or prolonged hospitalization	13	6
Bleeding requiring transfusion	*1*	*0*
Chest pain	*0*	*1*
Diarrhea	*0*	*1*
Hypotension and anemia	*1*	*0*
Meningitis and sepsis	*1*	*0*
Operative removal hematoma	*0*	*1*
Wound dehiscention requiring surgery	*1*	*0*
Pyelonephritis / urinary tract infection	*4*	*1*
Rectal prolaps with bleeding	*1*	*0*
Graft removal	*1*	*2*
Treatment for rejection	*2*	*0*
Wound infection	*1*	*0*
Medically significant	8	2
Acute coronary syndrome	*1*	*0*
Bleeding after surgery requiring two re-operations and intensive care admittance	*1*	*0*
Hemolysis	*1*	*0*
Hypotension, pulmonary edema, hemolysis and severe thrombocytopenia	*1*	*0*
Medication error	*0*	*2*

Severe infection	*2*	*0*
Urine leakage	*2*	*0*
Serious adverse events reported during administration of ATG-F		
Fever	1	0
Hypotension	4	0
Thrombocytopenia	3	0
Acute coronary syndrome due to blood loss / hypotension during transplantation	2	0

* P = 0.043, absolute risk difference 28%, 95%-CI 1% to 51%

the duration of delayed graft function did not differ from patients without infusion reactions (data not shown).

A difference in the type of donors (donation after brain death in most studies, compared to DCD in our study) also could explain the relatively high incidence of delayed graft function and the lack of a favorable effect of ATG-F. A lower incidence of delayed graft function was reported for patients treated with ATG-F in another study comparing ATG-F induction, basiliximab induction, or no induction (delayed graft function rate of 5.7%, 24.1%, and 15.9%; $p < 0.025$). In this study, however, only allografts from donation after brain death donors were included [126].

Kaden et al. reported that induction therapy with a single dose of ATG-F was correlated with a reduced incidence of delayed graft function, compared with a triple drug regimen with low cyclosporine dose (32.9% vs. 45.5%; $p < 0.01$) [127]. However, this was a retrospective study with potential bias. Other prospective studies evaluating the effect of a single intraoperative dose of ATG-F did not find a difference between patients treated with ATG-F, compared with a control group treated with either mycophenolate mofetil or standard dose cyclosporine [129, 133]. In the aforementioned studies, treatment with ATG-F was accompanied by a dose adjustment of the calcineurin inhibitor [126, 127]. As stated in our introduction, the combined use of ATG-F and adjustments of calcineurin inhibitors, makes it difficult to assess the separate effect of ATG-F. Because we used a regular, unadjusted tacrolimus dose and did not see an effect on the incidence of delayed graft function, the beneficial effect in other studies could possibly be caused by the dose adjustment of the calcineurin inhibitor, instead of the administration of anti-T-lymphocyte immunoglobulin. To investigate this hypothesis would require a study arm

with a reduced dose of the calcineurin inhibitor without ATG-F induction. This is not feasible, however, because one would expose the patient to an unjustifiable high risk of graft rejection.

Aside from the lack of efficacy, a higher incidence of serious adverse effects was reported in the ATG-F group. However, the open design of our study does not exclude bias, especially in reporting serious adverse events. Of the adverse effects occurring during and after administration of ATG-F, especially the thrombocytopenia and acute coronary syndrome, compromised patient safety. Another concern is the trend toward more infections in the ATG-F group, which was not reported in other studies with ATG-F. This could be either an effect of ATG-F itself, or related to the unadjusted, relatively higher (compared with other studies) tacrolimus dose in our study.

We prematurely terminated our study because of an unacceptable low recruitment rate. We initially aimed for the participation of seven Dutch transplant centers, but inclusion of study participants was initiated in only four of them. Moreover, the number of DCD donor renal allografts reported for transplant was smaller than estimated and more patients than expected met the exclusion criteria. Consequently, the study was underpowered to detect clinically meaningful differences in outcome parameters. However, based on the current findings with an incidence of delayed graft function of 79% in the ATG-F group and 54% in the control group, it is unlikely that expanding the study population from 54 to the planned number of 180 would yield a statistically significant benefit of ATG-F (the chance to achieve this was calculated to be 4%).

In conclusion, we are aware that our results must be considered with some caution, because our study was prematurely terminated. However, the intraoperative administration of a single high-dose of ATG-F in DCD donor renal allograft recipients, followed by triple immunosuppression with unadjusted tacrolimus dose, seems ineffective for reducing the incidence of delayed graft function. Because administration of ATG-F was associated with a higher rate of serious adverse events, the use of ATG-F in DCD donors to reduce the incidence of delayed graft function cannot be recommended.

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Chapter 5

Treatment of steroid-resistant acute renal allograft rejection with alemtuzumab

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Abstract

Steroid-resistant renal allograft rejections are commonly treated with rabbit antithymocyte globulin (RATG), but alemtuzumab could be an effective, safe and more convenient alternative.

Adult patients with steroid-resistant renal allograft rejection treated with alemtuzumab (15–30 mg s.c. on two subsequent days) from 2008 to 2012 (n = 11) were compared to patients treated with RATG (2.5-4.0 mg/kg bodyweight i.v. for 10–14 days; n = 20). We assessed treatment failure (graft loss, lack of improvement of graft function or need for additional antirejection treatment), infections during the first 3 months after treatment and infusion-related side effects.

In both groups, the median time-interval between rejection and transplantation was 2 weeks, and approximately 75% of rejections were classified as Banff IIA or higher. Three alemtuzumab-treated patients (27%) experienced treatment failure, compared to eight RATG treated patients (40%, p = 0.70). There was no difference in the incidence of infections. There were mild infusion-related side effects in three alemtuzumab treated patients (27%), and more severe infusion related side effects in 17 RATG-treated patients (85%, p = 0.013). Drug related costs of alemtuzumab treatment were lower than of RATG treatment (€1050 vs. €2024; p <0.01).

Alemtuzumab might be an effective therapy for steroid-resistant renal allograft rejections. In contrast to RATG, alemtuzumab is nearly devoid of infusion-related side effects. These data warrant a prospective trial.

Introduction

The first-line treatment of established acute cellular allograft rejection is high-dose steroids. When treatment with steroids fails or a rejection quickly recurs, treatment with anti-T cell antibodies is often the next step. Most experience is based on the use of antithymocyte globulin (ATG) and Muromonab-CD3 [134]. However, Muromonab-CD3 is no longer available and currently, steroid-resistant acute rejections are therefore mostly treated with a 10–14 days course of ATG [11, 13]. Although efficacious, this treatment has several drawbacks. First, ATG should be administered intravenously using a central venous catheter, high flow vein or arteriovenous fistula to prevent phlebitis. Second, the administration is associated with severe infusion-related side effects like fever, chills, headache, dyspnea, myalgia and hypotension [15]. This limits its tolerability, especially in older individuals or those with significant cardiopulmonary comorbidity.

Alemtuzumab is a depleting, humanized monoclonal antibody, directed specifically to the CD52 molecule, which is expressed on T cells and several other lymphoid and myeloid cell types [21]. Alemtuzumab is currently registered for the treatment of chronic lymphatic leukemia, but data on the safety and efficacy of treatment of acute rejection after organ transplantation are scarce. Several studies reported the results in small groups of patients, merely demonstrating that recurrent or steroid-resistant rejection can be reversed with alemtuzumab [30, 31, 34-36]. Randomized trials comparing alemtuzumab with other T cell-depleting agents for the treatment of acute rejection have not been performed. In early trials with alemtuzumab as induction therapy, intravenous administration was frequently accompanied by infusion reactions. In more recent trials, in which alemtuzumab was given subcutaneously, infusion-related side effects were rare or not reported [38, 63].

We hypothesized that alemtuzumab might be preferred over ATG for steroid-resistant rejection after renal transplantation. We analyzed our first experience with alemtuzumab in this setting, and compared this with results obtained in a historical cohort of patients treated with ATG.

Patients and Methods

All renal allograft recipients treated with alemtuzumab for steroid-resistant rejection since 2008 were identified in three academic centers in the Netherlands. Subsequently, a control group was composed, consisting of patients that were treated with ATG for

steroid-resistant acute rejection. For each alemtuzumab-treated patient, we selected the two ATG-treated patients in the same center of whom the transplantation date was most closely preceding and following that of the alemtuzumab-treated patient.

Next, we excluded patients who never had a functioning graft (defined as urine production posttransplantation with a drop in serum creatinine without requirement of dialysis) before the onset of antibody therapy, or in whom there was no biopsy confirming the presence of acute cellular rejection. In addition, patients who already had received any anti-T cell antibodies after the current transplantation (as induction therapy or treatment of prior rejection episode) were excluded.

Consequently, none of the analyzed patients had received induction therapy with a depleting anti-T cell agent. Some patients received basiliximab as induction therapy, and another part of the patients participated in an ongoing double-blind randomized trial, comparing rituximab with placebo as induction therapy. Maintenance immunosuppression consisted of the combination of a calcineurin inhibitor, an antiproliferative agent, and steroids in all patients. In case of graft dysfunction without obvious pre- or postrenal cause, a graft biopsy was performed. Pathologic examination of the biopsy tissue included C4d staining in all cases and rejections were classified according to Banff 97 criteria [10]. When a graft biopsy was not possible (e.g. because of the use of anticoagulant therapy) and the suspicion of rejection was strong, antirejection therapy was started without prior graft biopsy. Donor-specific anti-HLA antibodies were not routinely measured. First-line antirejection treatment consisted of methylprednisolone, with doses varying between 500 and 1000 mg during 3–6 days. When there was no successful response to steroids, or graft function deteriorated during or shortly after steroid therapy, the rejection episode was considered steroid-resistant. In part of the cases a (re)biopsy was performed to confirm the persistence of rejection. Alemtuzumab (Campath-1H Genzyme Europe BV, Naarden, The Netherlands) was given in 15–30 mg doses subcutaneously at two subsequent days. ATG (Thymoglobulin, Genzyme) was administered in a dosage of 2.5–4.0 mg/kg bodyweight intravenously for 10–14 days, with adjustments based on lymphocyte or CD4+ T cell counts. In both groups patients were treated with antihistamines, steroids and acetaminophen before antibody therapy.

We assessed the rate of treatment-failure and infections during the first 3 months after the start of treatment with anti-T cell antibodies, and recorded all malignancies during follow-up. Treatment failure was defined as graft loss, the need for additional

antirejection therapy or lack of improvement of graft function (defined as the absence of a drop in serum creatinine of 25% or more at any time within a 3 month interval after start of treatment). Furthermore, we analyzed the incidence of infusion-related side effects and the drug-related costs of both antibody treatments.

We performed overall group comparisons using a chi-square test or Fisher exact test (if counts per group were below five). For continuous variables we used either an unpaired T test (normally distributed data) or a Wilcoxon test (not-normally distributed data). All statistical tests were two-sided and $p < 0.05$ was considered statistically significant.

Results

From January 2008 to April 2012, we identified 14 patients treated with alemtuzumab and 28 patients treated with ATG. Of the 14 alemtuzumab-treated patients, two were treated earlier with other anti-T cell antibodies (muromonab-CD3 and ATG, respectively) after the current transplantation, and one was still on dialysis at start of antibody treatment. Therefore, 11 alemtuzumab-treated patients were included in the analysis. In five patients in the control group, no biopsy was performed to confirm the presence of rejection, and in one case there was only borderline rejection. Moreover, one patient had received ATG as induction therapy, and one was still on dialysis at start of antibody treatment. As a result, 20 ATG-treated patients were analyzed.

In the majority of alemtuzumab-treated patients, the use of other anti-T cell antibodies was considered to be unattractive for the following reasons: treatment with ATG after a previous transplantation (n = 4), positive test for antirabbit IgG antibodies (n = 2), fluid overload (n = 1) and recent cardiac ischemia (n = 1). In three cases, alemtuzumab was chosen without specific contra-indication for other anti-T cell antibodies.

Baseline characteristics of alemtuzumab- and ATG-treated patients are stated in **Table 1**. The number of HLA mismatches was lower in the alemtuzumab-treated patients. However, 55% of the alemtuzumab-treated patients underwent a retransplant compared to only 5% of the ATG-treated patients ($p < 0.01$). The majority of rejections in each group were classified as Banff IIA. The median time interval between transplantation and antibody treatment of rejection was 42 days in the alemtuzumab group (range 4–752) and 22 days in ATG-treated patients (range 6–849; $p = 0.73$).

Three alemtuzumab-treated patients (27%) experienced treatment failure, compared with eight ATG-treated patients (40%; p = 0.70), see **Tables 2 and 3**. Additional information on individual patients may be found in **Tables 4 and 5**. Serum creatinine 3 months after start of antibody treatment was comparable between both groups (182 ± 84 vs. 187 ± 101 μmol/L; p = 0.89). The median number of infections per patient within 3 months after treatment was one in alemtuzumab-treated patients (range 0–6) and two in ATG-treated patients (range 0–6; p = 0.81). A cytomegalovirus (CMV) primary infection or reactivation occurred in four alemtuzumab-treated patients, compared to five among ATG-treated patients (36% vs. 25%; p = 0.68).

Table 1. Baseline characteristics of patients treated with alemtuzumab or ATG.

	Alemtuzumab (n = 11)	**ATG (n = 20)**	**p values**
Recipient age, years (median, range)	49 (19 – 72)	48 (24 – 66)	0.93
Sex – no. (%)			0.48
Male	5 (45)	12 (60)	
Female	6 (55)	8 (40)	
Cause of end-stage renal disease – no. (%)			0.32
Glomerulonephritis	6 (55)	6 (30)	
Polycystic kidney disease	2 (18)	3 (15)	
Diabetic nephropathy	0 (0)	2 (10)	
Hypertension	1 (9)	1 (5)	
Other	2 (18)	8 (40)	
Rank order of transplantation – no. (%)			<0.01
First	5 (45)	19 (95)	
Second or more	6 (55)	1 (5)	
Peak value of panel reactive antibodies % (median, range)	10 (0 – 83)	0 (0 – 92)	0.077
HLA A, B, and DR mismatches – no. (median, range)	2 (1 – 5)	5 (1 – 6)	0.029
Donor age, years (median, range)	51 (10 – 74)	53 (36 – 66)	0.89
Type of donor – no. (%)			0.32
Donation after brain death	5 (45)	4 (20)	
Donation after circulatory death	1 (9)	2 (10)	
Living	5 (45)	14 (70)	

During follow-up, two ATG-treated patients developed a malignancy (one lymphoma at 35 months and one squamous-cell carcinoma at 30 months after treatment). One or more infusion-related side effects occurred in three alemtuzumab-treated patients (27%). One patient developed malaise, dyspnea, myalgia and tachycardia, whereas the other two had either a local hematoma or a mild rash. In comparison, all but three ATG-treated patients experienced one or more infusion-related side effects such as hypotension, fever, chills, nausea, malaise and thrombocytopenia (85%; $p = 0.013$). The median drug-related costs of alemtuzumab treatment were €1050 (range €525–€1050), whereas the median drug-related costs of ATG treatment were €2024 (range €855–€3875; $p < 0.01$).

Table 2. Outcome in alemtuzumab-treated patients.

ID	Lowest serum creatinine posttransplantation (µmol/l)	Pre-treatment serum creatinine (µmol/l)	Three months post-treatment serum creatinine (µmol/l)	Efficacy within three months after treatment		
				Graft loss	Lack of improvement of graft function	Need for further antirejection treatment
1	584	781	247	No	No	No
2	86	128	Patient died	Yes	Yes	No
3	331	332	200	No	No	No
4	94	132	87	No	No	No
5	603	658	165	No	No	No
6	52	77	76	No	No	No
7	523	889	255	No	No	No
8	96	486	350	No	No	No
9	96	138	131	No	Yes	No
10	97	131	140	No	Yes	No
11	110	166	171	No	No	No

Table 3. Outcome in ATG-treated patients.

ID	Lowest serum creatinine posttransplantation (µmol/l)	Pre-treatment creatinine (µmol/l)	Three months post-treatment creatinine (µmol/l)	Efficacy within three months after treatment		
				Graft loss	Lack of improvement of graft function	Need for further antirejection treatment
12	246	345	108	No	No	No
13	325	896	148	No	No	No
14	468	562	158	No	No	No
15	123	350	121	No	No	No
16	171	278	102	No	No	No
17	286	468	217	No	No	No
18	112	490	181	No	No	Yes
19	164	164	95	No	No	No
20	120	126	130	No	Yes	No
21	214	306	141	No	No	No
22	105	350	413	No	Yes	No
23	118	285	Patient died	Patient died	No	No
24	119	158	100	No	No	No
25	153	164	141	No	No	No
26	484	602	220	No	No	no
27	245	485	264	No	No	Yes
28	611	608	290	No	No	No
29	168	224	238	No	Yes	No
30	152	219	516	Yes	Yes	No
31	94	301	201	No	No	Yes

Table 4. Individual patient data on immunosuppression, graft histology and timing of rejections in alemtuzumab-treated patients.

ID	Immunosuppression (Induction / maintenance)	Interval between transplantation and start of steroid treatment (days)	Rejection type and C4d staining before steroid treatment	Interval between transplantation and start of antibody treatment (days)	Rejection type and C4d staining before antibody treatment
1	Basiliximab / tacrolimus, MMF, steroids	2	IIA, C4d negative	4	No rebiopsy performed
2	Rituximab or placebo/ cyclosporine, MMF, steroids	33	IB, C4d negative	42	No rebiopsy performed
3	Rituximab or placebo/ tacrolimus, MMF, steroids	7	No biopsy performed	16	IIA, C4d negative
4	Rituximab or placebo/ tacrolimus, MMF, steroids	6	IIA, C4d positive	15	IIA, C4d negative
5	Rituximab or placebo/ tacrolimus, MMF, steroids	10	IIA, C4d positive	18	IIA, C4d negative
6	Basiliximab / cyclosporine, azathioprin, steroids	341	IIA, C4d negative	351	IIA, C4d negative
7	Basiliximab / cyclosporine, MMF, steroids	2	IIA, C4d negative	4	No rebiopsy performed
8	Basiliximab / tacrolimus, MMF, steroids	258	IA, C4d positive	271	No rebiopsy performed
9	Basiliximab / tacrolimus, azathioprin, steroids	745	IB, C4d negative	752	No rebiopsy performed
10	Basiliximab / tacrolimus, MMF, steroids	52	No biopsy performed	71	IIA, C4d negative
11	None / tacrolimus, MMF, steroids	56	IIA, C4d negative	81*	IIA, C4d negative

* After two separate antirejection treatments with methylprednisolone a third biopsy showed ongoing rejection, for which alemtuzumab was given without a preceding course of methylprednisolone. MMF: mycophenolate mofetil

Table 5. Individual patient data on immunosuppression, graft histology and timing of rejections in ATG-treated patients

ID	Immunosuppression (Induction / maintenance)	Interval between transplantation and start of steroid treatment (days)	Rejection type and C4d staining before steroid treatment	Interval between transplantation and start of antibody treatment (days)	Rejection type and C4d staining before antibody treatment
12	None / tacrolimus, MMF, steroids	5	No biopsy performed	10	IIA, C4d negative
13	None/ tacrolimus, MMF, steroids	6	No biopsy performed	13	IB, C4d negative
14	Rituximab or placebo/ tacrolimus, MMF, steroids	3	IIA, C4d positive	6	No rebiopsy performed
15	Rituximab or placebo/ tacrolimus, MMF, steroids	5	No biopsy performed	8	IIA, C4d positive
16	None / cyclosporine, MMF, steroids	19	IIA, C4d positive	21	No rebiopsy performed
17	Rituximab or placebo/ tacrolimus, azathioprine, steroids	23	IIA, C4d negative	77*	IA, C4d negative
18	Rituximab or placebo/ tacrolimus, azathioprine, steroids	217	IB, C4d negative	219	No rebiopsy performed
19	Rituximab or placebo/ tacrolimus, MMF, steroids	7	IIB, C4d negative	26	IIA, C4d negative
20	None / cyclosporine, MMF, steroids	11	IIA, C4d positive	22	IIA, C4d positive
21	Basiliximab / MMF, steroids	6	No biopsy performed	15	IIA, C4d negative
22	Basiliximab / MMF, steroids	847	IIB, C4d negative	849	No rebiopsy performed
23	Basiliximab / tacrolimus, MMF, steroids	170	IIA, C4d negative	172	No rebiopsy performed
24	None / tacrolimus, MMF, steroids	5	IIA, C4d negative	9	No rebiopsy performed
25	Rituximab or placebo/ tacrolimus, MMF, steroids	98	IB, C4d negative	126**	IIA, C4d negative
26	None / tacrolimus, MMF, steroids	7	IA, C4d positive	17	No rebiopsy performed
27	Rituximab or placebo/ tacrolimus, MMF, steroids	4	No biopsy performed	9	IIA, C4d negative
28	Basiliximab / tacrolimus, MMF, steroids	7	IIA, C4d negative	9	No rebiopsy performed

29	Basiliximab / tacrolimus, MMF, steroids	207	IIA, C4d positive	217	No rebiopsy performed
30	Basiliximab / everolimus, MMF, steroids	97	IA, C4d negative	113	IB, C4d negative
31	Basiliximab / tacrolimus, MMF, steroids	210	No biopsy performed	225	IB, C4d negative

* Patient had been treated with methylprednisolone twice for two separate periods of rejection; despite this a third episode of rejection occurred, which was directly treated with ATG.

** Three weeks after a treatment with methylprednisolone, an even worse rejection (type IIA, compared to IB) was diagnosed. Serum creatinine had not dropped significantly in the meantime. Therefore, this was considered a steroid-resistant rejection and ATG treatment was started. MMF: mycophenolate mofetil

Discussion

In this retrospective analysis, alemtuzumab appeared to be similarly effective as ATG in the treatment of steroid-resistant renal allograft rejection. The incidence of infections was low and not different from that seen after ATG therapy. Importantly, treatment with alemtuzumab was associated with fewer infusion-related side effects that were also considerably milder.

These results have to be interpreted with caution however, because of the nonrandomized and small nature of this study. Of note, there were some differences between both groups. There were more retransplanted patients in the alemtuzumab group, which could partly be explained by the fact that in a number of cases alemtuzumab was used to avoid repeated ATG treatment after prior use for an earlier graft. In general, retransplanted patients may be more sensitized and therefore more difficult to treat. However, despite the larger number of retransplants in the alemtuzumab group, the outcome was similar to that in the ATG-treated patients. Moreover, we noticed that within both groups there was considerable variation in the interval between transplantation and rejection. No difference in effectiveness of alemtuzumab was found in early versus late (defined as occurring more than 6 months posttransplant) rejections (data not shown), but the small numbers in these subgroups preclude firm conclusions.

The incidence of infections was similar in both groups, including the incidence of primary CMV infection of reactivation. However, our follow-up period was relatively short, especially considering the long-lasting T cell-depletion that can be observed after alemtuzumab treatment [22]. The majority of literature data concern the use of alemtuzumab as induction therapy. In this regard, it is of interest that one of the trials on induction therapy with alemtuzumab showed less acute rejections but an increased incidence of CMV disease [50]. This was not confirmed in a larger cohort of 547 patients [51]. However, the latter study showed that the incidence of opportunistic infections was increased when patients received alemtuzumab for the treatment of allograft rejection compared with those who received alemtuzumab as induction therapy (21 vs. 4.5%; $p < 0.01$). In a noncomparative study with 40 patients treated with alemtuzumab for steroid-resistant or severe rejection, a total of 14 patients (35%) had infectious complications, of whom two (5%) died [31]. In a recent article reporting long-term follow-up data, the Cambridge group also found an excess of early infection-related death in alemtuzumab-treated patients [30].

Aside from these concerns, our data show great advantages of the use of alemtuzumab, namely the significantly lower incidence of infusion-related side effects, the possibility to administer alemtuzumab subcutaneously and lower costs. This renders alemtuzumab a valuable alternative for ATG to treat acute rejection, especially in frail patients. We think that a randomized prospective trial to compare these two treatments is warranted.

Chapter 6

Rituximab as induction therapy after renal transplantation: a randomized, double-blind, placebo-controlled study of efficacy and safety

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Abstract

We evaluated the efficacy and safety of rituximab as induction therapy in renal transplant patients. In a double-blind, placebo-controlled study, 280 adult renal transplant patients were randomized between a single dose of rituximab (375 mg/m^2) or placebo during transplant surgery. Patients were stratified according to panel reactive antibody (PRA) value and rank number of transplantation. Maintenance immunosuppression consisted of tacrolimus, mycophenolate mofetil, and steroids. The primary endpoint was the incidence of biopsy-proven acute rejection (BPAR) within six months after transplantation.

The incidence of BPAR was comparable between rituximab-treated (23/138, 16.7%) and placebo-treated patients (30/142, 21.2%, p = 0.25). Immunologically high-risk patients (PRA >6% or retransplant) not receiving rituximab had a significantly higher incidence of rejection (13/34, 38.2%) compared to other treatment groups (rituximab-treated immunologically high-risk patients, and rituximab- or placebo-treated immunologically low-risk (PRA 6% or first transplant) patients (17.9%, 16.4%, and 15.7%, p = 0.004). Neutropenia (<1.5 10^9/L) occurred more frequently in rituximab-treated patients (24.3% vs. 2.2%, p < 0.001). After 24 months, the cumulative incidence of infections and malignancies was comparable.

A single dose of rituximab as induction therapy did not reduce the overall incidence of BPAR, but might be beneficial in immunologically high-risk patients. Treatment with rituximab was safe.

Introduction

With the combination of a calcineurin inhibitor, mycophenolate mofetil, and prednisolone as immunosuppressive treatment, the incidence of acute rejection after renal transplantation is acceptably low. Since acute rejection is one of the main predictors of chronic transplant glomerulopathy, further lowering of incidence of acute rejection, e.g. by the additional use of IL-2 receptor antagonists or polyclonal anti-T cell antibodies, might improve long-term outcome [85, 135, 136]. Increased attention for the role of B cells and antibodies in acute rejection has been elicited by the negative prognostic impact of donor-specific anti-HLA antibodies, the presence of B cell clusters in biopsies of patients with severe rejection, and the frequent finding of capillary deposition of C4d in patients with acute rejection [2, 3]. B cells are the progenitors of plasma cells, are effective antigen presenting cells, and can secrete different cytokines to stimulate cellular immunity. Interfering with these pathways by anti-B cell therapy has been shown to be effective in diseases that were considered to be mainly T cell driven, like rheumatoid arthritis [4, 5].

Based on these considerations, we chose to investigate the effectiveness of the anti-B cell monoclonal antibody rituximab as induction therapy after renal transplantation. Rituximab induces long-lasting B cell depletion in peripheral blood with limited short- and long-term toxicity [110, 137]. Most experience with rituximab in renal transplantation stems from its use in ABO-incompatible transplantation, where low rates of acute rejection were observed [96]. At time of initiation of the current study, no data were available on the effect of rituximab on acute rejection in ABO-compatible transplantation. We tested the hypothesis that adding a single dose of rituximab to an immunosuppressive regimen consisting of tacrolimus, mycophenolate mofetil and steroids would reduce the incidence of biopsy-proven acute renal allograft rejection (BPAR).

Methods

We performed a single center, randomized, double-blind, placebo-controlled study at the Radboud University Medical Center, Nijmegen, The Netherlands, from December 2007 to June 2012. All patients of 18 years or older who were scheduled to receive a renal allograft from either a living or deceased ABO compatible donor were screened for eligibility by the nephrologist on call. To be included, the immunosuppressive treatment had to consist of a combination of tacrolimus, mycophenolate mofetil, and prednisolone. At the time of design of the study, induction therapy with IL-2 receptor antagonists or anti T cell antibodies was not

part of our hospital protocol, and was therefore not used in this trial. Other exclusion criteria were: a HLA-identical living donor; hemolytic uremic syndrome as original kidney disease; focal segmental glomerulosclerosis that had recurred in a previous graft; three or more previously failed grafts; a current or historic panel reactive antibody (PRA) value 85%; total white blood cell count <3.0 10^9/L; platelet count <75 10^9/L; active infection with hepatitis B, hepatitis C, or HIV; a history of tuberculosis; and previous treatment with rituximab. Female patients at risk for pregnancy had a negative serum pregnancy test before randomization and agreed to use contraception for 12 months. The PRA value was defined as the percentage of panel cells that reacts with patient serum in the complement-dependent cytotoxicity screening. The panel cells consisted of lymphocyte suspensions obtained from 60 different healthy individuals selected for HLA A, B, DR, and DQ as to achieve a maximum ability to detect anti-HLA antibodies [138].

All patients had negative B- and T cell complement-dependent lymphocytotoxic cross-matches with current and historic sera at time of transplantation. All patients provided written informed consent before study entry. The study was approved by the Committee on Human-Related Research Arnhem–Nijmegen, conducted according to the Declaration of Helsinki and good clinical practice guidelines, and reported according to CONSORT guidelines [139].

Patients were randomized in a 1:1 ratio to treatment with rituximab or placebo. Since we hypothesized that the effects of rituximab could be unequal in immunologically high- and low-risk patients, we stratified upfront for PRA according to the Eurotransplant cut-off value of 6% for allo-sensitization, and history of prior transplantation (first vs. retransplant). For allocation, a computer-generated list of random numbers was used for each of the four strata, prepared by an independent investigator. This list containing study number and treatment allocation was only accessible for authorized nurses, who signed confidentiality statements. For every new included patient, the lowest available study number was handed to one of the authorized nurses, who prepared study medication according to the randomization list.

Patients randomized to rituximab received a single dose of 375 mg/m^2 intravenously during surgery. The required dose was diluted in a 500 ml bag of 0.9% sodium chloride. In placebo-treated patients infusion consisted of an identical 500 ml bag. Both bags had an identical appearance and were labeled ‘study medication’.

At the start of the operation patients received standard antibiotic prophylaxis next to 100 mg prednisolone and 2 mg clemastine intravenously to prevent allergic reactions to rituximab. Infusion of study medication was started 30 minutes thereafter at a rate of 60 ml/hour, increased every 30 minutes to a maximum rate of 200 ml/hour.

After the operation prednisolone was continued intravenously for three days at 100 mg/day, followed by 15–25 mg/day prednisolone orally, according to bodyweight, and tapered to 0.1 mg/kg/day. Tacrolimus (Prograft, Astellas Pharma) was given in a dose of 0.2 mg/kg/day, divided in two doses. The target trough levels were 15–20 ng/ml in the first two weeks posttransplant, 10–15 ng/ml during weeks 3–6, and 5–10 ng/ml thereafter. Mycophenolate mofetil (CellCept, Hoffman-La Roche) was started at 2000 mg/day, divided in two doses, and reduced to 1500 mg/day after two weeks unless patients weighed more than 90 kg. Additional treatment consisted of trimethoprim/sulfamethoxazole 480 mg/day for the first 3 months, and thrice weekly thereafter until one year posttransplant. Cytomegalovirus (CMV) seronegative patients who received a kidney from a CMV seropositive donor were treated prophylactically with valganciclovir during the first three months. Additionally, valganciclovir prophylaxis was given for two months after treatment with anti-T cell antibodies when either the donor or recipient was CMV seropositive.

First-line antirejection therapy consisted of methylprednisolone for three consecutive days in a dose of 500-1000 mg/day intravenously. Steroid-resistant rejections were treated with anti-T cell antibodies (Rabbit anti-thymocyte globulin [Thymoglobulin], Genzyme; Muromonab-CD3 [OKT3], Janssen-Cilag; alemtuzumab [Campath], Genzyme) according to local practice. Rejection was considered steroid-resistant if no stabilization or improvement of graft function occurred within five days after the first methylprednisolone dose.

Efficacy and safety

The primary end point was the incidence and severity of BPAR within the first six months after transplantation. For patients with more than one biopsy available during a single rejection episode, the biopsy-score with the highest Banff grade was used for analysis. Borderline rejections were excluded. Biopsies were scored independently by two blinded pathologists according to the updated Banff 07 criteria [140]. In case of different conclusions, biopsies were re-evaluated collectively. Protocol graft biopsies were not performed.

Secondary end points included the estimated glomerular filtration rate (eGFR) at six months in patients with a functioning graft, cumulative incidence of infections and malignancies at six and 24 months, and patient and graft survival at six months and at end of follow-up. All serious adverse events were recorded during 24 months.

Number and phenotype of B cells

Blood was taken immediately before transplantation. Peripheral blood mononuclear cells (PBMCs) were isolated by gradient centrifugation using Lymphoprep (Lucron, Dieren, The Netherlands) and stored in liquid nitrogen until further use. Cell phenotypes were analyzed by 10-color flow cytometry (Navios, Beckman-Coulter, Fullerton, USA). The following fluorochrome-conjugated monoclonal antibodies were used to study B cells: CD19(J3-119)-APC Alexa Fluor 750, CD24(ALB9), CD27(1A4-CD27)-PeCy5.5, CD38(L498-4-3), CD45(J.33)-Krome Orange and IgD(IADB6)-FITC (Beckman-Coulter). Isotype controls or unstained cells were used for gate settings. Data were analyzed using Kaluza software (Beckman-Coulter).

The B cell phenotype was analyzed in immunologically high-risk patients (for definition see below), based on availability of the samples (n = 26). For comparison we selected 28 immunologically low-risk matched for age, gender, type of dialysis, and CMV status.

Statistical analysis

The primary efficacy endpoint was biopsy confirmed acute rejection within the first six months after transplantation. Trials performed before the start of our study showed an incidence of this endpoint of about 15% in patients treated with tacrolimus, mycophenolate mofetil and steroids. Although the benefit of additional rituximab treatment was not known (no literature data at the time of trial design), using data concerning ABO-incompatible transplantations, we assumed that the incidence of biopsy-proven rejection could drop to about 5% [96]. To detect a decrease in rejection incidence from 15% to 5% with a two-sided 5% significance level and a power of 80%, the required sample size was 140 patients per treatment arm. The trial was not powered to test superiority in the different strata. After 70 patients had reached a follow-up of six months, a planned interim safety analysis was performed to test the cumulative incidence of infections and malignancies [141]. This interim analysis was not performed to test efficacy or futility.

Statistical testing was performed according to distribution and type of data (unpaired T test, Mann-Whitney U, or Fisher's exact tests). Time to first BPAR, allograft loss, and death were analyzed with the Kaplan–Meier method, and differences were assessed by the log-rank test.

The four pre-specified strata were grouped as follows: patients with a retransplant or PRA value >6% were considered to be immunologically high-risk, and those with a first transplant and PRA value 6% were regarded as immunologically low-risk. Comparison of different

immunological risk groups was performed by one-way ANOVA, Kruskal-Wallis, and chi-square tests. All data were analyzed on an intention-to-treat basis. Analyses were performed with IBM SPSS Advanced Statistics 20.0. This study is registered with ClinicalTrials.gov register, number NCT00565331.

Results

Between December 2007 and June 2012, 488 adult renal transplant candidates were evaluated for eligibility and 281 patients were included, of whom 139 patients were randomized to rituximab and 142 to placebo (**Figure 1**). One patient did not undergo transplantation and was therefore excluded from all analyses. Overall, the groups were well balanced with respect to demographic, clinical, and donor characteristics (**Table 1**).

Infusion with rituximab was well tolerated and all but six patients received the full dose (Figure 1). One patient experienced an anaphylactic reaction during surgery, which was attributed to rituximab. She recovered uneventfully. Temporary interruption of the infusion, mainly due to hypotension, occurred in seven rituximab-treated patients (5.1%) compared to five placebo-treated patients (3.5%, $p = 0.57$ by Fisher's exact test).

Analysis of B cells in peripheral blood in 20 CMV-negative patients without BPAR, confirmed nearly complete depletion in rituximab-treated patients as compared to placebo-treated patients at six months after transplantation (median CD19+ B cells and range; 0.6/µl (0/µl – 16.4/µl) vs. 141/µl (31/µl – 458/µl); $p < 0.001$). The primary outcome, BPAR within six months after transplantation, occurred in 23 of the 138 rituximab-treated patients (16.7%), compared to 30 of 142 placebo-treated patients (21.1%, $p = 0.25$ by log-rank test, **Figure 2A**). Based on the pre-specified stratification according to PRA value and rank number of transplantation, we grouped the four strata to form an immunologically low-risk group (n = 218) and an immunologically high-risk group (n = 62). Immunologically high-risk patients receiving placebo had a significantly higher incidence of acute rejection within the first six months compared to immunologically low-risk patients (rituximab- or placebo-treated) and rituximab-treated immunologically high-risk patients (38.2% vs. 16.4%, 15.7%, and 17.9%, $p = 0.004$ by log-rank test, **Figure 2B**). This effect persisted at two years after transplantation (data not shown). When the group of immunologically high-risk patients was analyzed separately, there was a clear trend towards a lower incidence of BPAR with rituximab treatment as compared to placebo (17.9% vs. 38.2% during the first six months, $p = 0.06$ by log-rank test).

Figure 1. Trial profile of all patients.

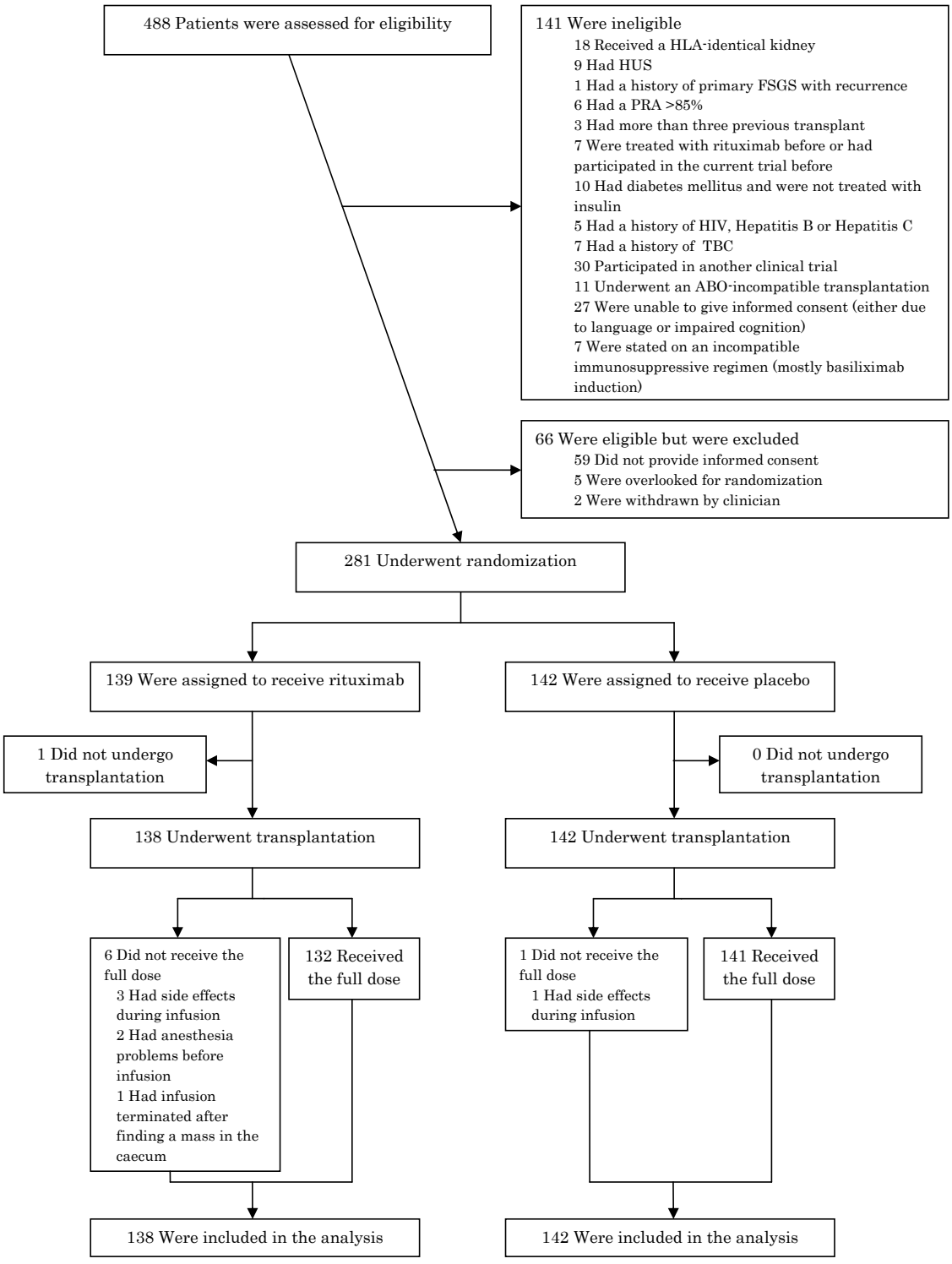


Table 1. Baseline characteristics of the patients*

Variable	All patients		Immunologically low-risk patients		Immunologically high-risk patients	
	Rituximab (n = 138)	Placebo (n = 142)	Rituximab (n = 110)	Placebo (n = 108)	Rituximab (n = 28)	Placebo (n = 34)
Age (yr)	50.8 ± 13.2	49.8 ± 12.3	51.0 ± 13.6	50.6 ± 11.8	49.8 ± 11.7	47.3 ± 13.6
Male sex (%) †	69.6	63.4	71.8	69.4	60.7	44.1
White race (%) ‡	94.9	96.5	94.5	96.3	96.4	97.1
Cause of end-stage renal disease (%)						
Glomerulonephritis	29.0	34.5	28.2	33.3	32.1	38.2
Diabetes mellitus	4.3	6.3	4.5	5.6	3.6	8.8
Urological disorder	7.2	7.0	7.3	5.6	7.1	11.8
Hypertension / vascular damage	6.5	7.0	6.4	7.4	7.1	5.9
Polycystic kidney disease	22.5	22.5	22.7	26.9	21.4	8.8
Uncertain	15.9	14.8	16.4	13.0	14.3	20.6
Other	14.5	7.7	14.5	8.4	14.3	5.9
Type of donor (%)						
Living	58.7	57.0	60.9	61.1	50.0	44.1
Deceased – donation after circulatory death	10.1	13.4	10.0	11.1	10.7	20.6
Deceased – donation after brain death	31.2	29.6	29.1	27.8	39.3	35.3
Donor age (yr)	54.1 ± 11.6	52.8 ± 10.2	54.7 ± 11.4	53.4 ± 9.1	51.6 ± 12.1	50.7 ± 13.0
Antigen mismatches — A, B, and DR (no.)	3.30 ± 1.62	3.15 ± 1.46	3.26 ± 1.60	3.21 ± 1.49	3.43 ± 1.77	2.94 ± 1.37
Panel reactive antibody titer — highest assessment§	0 (0 – 83)	0 (0 – 71)	0 (0 – 6)	0 (0 – 5)	17 (2 – 83)	15 (0 – 71)

Patients with retransplant (%)	8.7	10.6	-	-	42.9	44.1
Cold-ischemia time — deceased donors only (hr)	17.2 ± 5.1	17.4 ± 5.5	16.7 ± 5.2	17.0 ± 5.3	18.8 ± 4.7	18.3 ± 6.0
Cytomegalovirus serologic status — donor positive, recipient negative (%)	21.7	23.2	21.8	26.9	21.4	11.8

* Values are presented as mean ± standard deviation or median (range). Overall group differences were not significant.

† $p = 0.02$ for all strata calculated with the chi-square test.

‡ Race was determined by the investigator.

§ $p < 0.01$ for all strata calculated with the chi-square test.

Since our data suggested that a potential effect of rituximab was limited to immunologically high-risk patients, we compared B cell number and phenotype in pre-transplant blood samples of immunologically high and low-risk patients. Interestingly, the number of CD19+ B cells, especially CD27+ memory B cells, was higher in the immunologically high-risk patients (CD19+CD27+ memory B cells 35 cells/µl [12-152] vs. 24 cells/µl [5-78], p = 0.02 by Mann-Whitney U test, **Figure 3**). The number of CD24++CD38++ transitional B cells was comparable in both patient groups.

Most rejections were T cell mediated according to the Banff classification (**Table 2**). Rituximab-treated patients tended to have less antibody mediated rejections (ABMR), compared to placebo-treated patients (4/138, 2.9% vs. 11/142, 7.7% p = 0.11 by Fisher's exact test). At six months, the dose of mycophenolate mofetil and trough level of tacrolimus were somewhat lower in the rituximab-treated patients (**Table 3**).

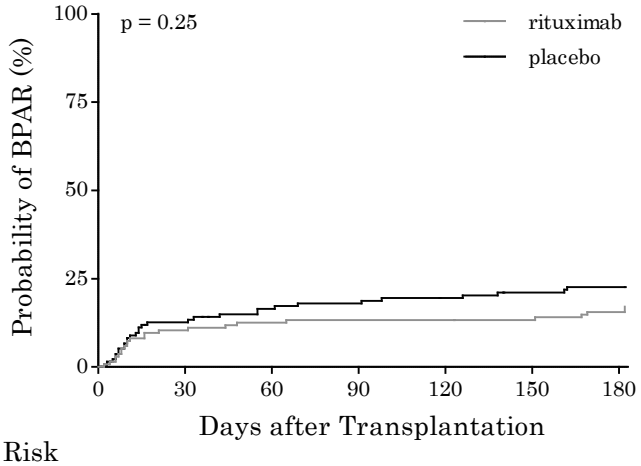
Patient and graft survival (at six months, and after a median duration of follow-up of 4.0 years, range 1.9 – 6.4 years) as well as graft function and proteinuria (at six months and at 24 months) were comparable between the rituximab and placebo group (**Table 4**).

The incidence of delayed graft function did not differ between rituximab- and placebo-treated patients, but within the immunologically high-risk subgroup, a trend towards a lower incidence was seen in rituximab-treated patients (5/28, 17.9% vs. 12/34, 35.3% p = 0.13 by chi-square test).

One rituximab-treated patient was diagnosed with progressive multifocal leukoencephalopathy and died shortly thereafter. The overall incidence of infections or malignancies was not higher after treatment with rituximab compared to placebo. During the first six months after transplantation, treatment with rituximab was associated with a significantly higher cumulative incidence of grade 2 or more severe leucopenia (19.0% vs. 1.4%, p < 0.001 by Fisher's exact test) and neutropenia (24.3% vs. 2.2%, p < 0.001 by Fisher's exact test, **Table 4**). Consequently, the number of patients in whom mycophenolate mofetil was (temporarily) discontinued during the first six months tended to be higher in the rituximab group than in the placebo group (18/131 vs. 8/128, p = 0.06 by Fisher's exact test), but interruption of treatment with this drug was not correlated with the occurrence of acute rejection.

Figure 2A. Cumulative probability of biopsy-proven acute rejection in all patients.

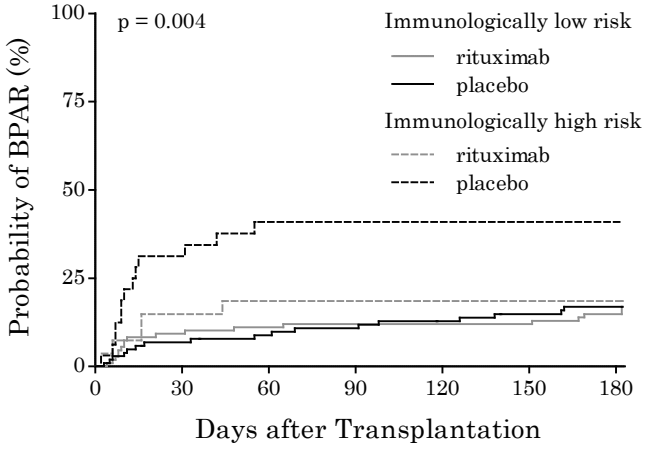
A



Number at Risk

Rituximab	138	121	118	117	117	116	113
Placebo	142	116	109	107	104	101	99

B



Number at Risk

Immunologically low risk, rituximab	110	98	96	95	95	95	92
Immunologically low risk, placebo	108	94	91	89	86	83	81
Immunologically high risk, rituximab	28	23	22	22	22	21	21
Immunologically high risk, placebo	34	22	18	18	18	18	18

Table 2. Incidence and type of biopsy-proven acute rejection (BPAR) at six months*

Variable	All patients		p value	Immunologically low-risk patients		Immunologically high-risk patients		p value
	Rituximab (n = 138)	Placebo (n = 142)		Rituximab (n = 110)	Placebo (n = 108)	Rituximab (n = 28)	Placebo (n = 34)	
Patients with biopsy-proven rejection (no. – %)	23 (16.7)	30 (21.1)	0.25	18 (16.4)	17 (15.7)	5 (17.9)	13 (38.2)	0.004
Patients with steroid-resistant biopsy-proven rejection (no. – % of biopsy-proven rejections)	10 (43.5)	15 (50.0)	0.66	6 (33.3)	6 (35.3)	4 (80.0)	9 (69.2)	0.009
T cell mediated rejection (no.)								
Type IA	5	6		5	6	0	0	
Type IB	2	2		2	2	0	0	
Type IIA	10	9		7	6	3	3	
Type IIB	2	2		2	0	0	2	
Antibody mediated rejection (ABMR; no.)	1	2		1	1	0	1	
Combined rejections (no.)								
ABMR + Type IIA	2	9		1	2	1	7	
ABMR + Type IIB	1	0		0	0	1	0	

* Biopsies were independently scored by two pathologists according to the Banff 07 classification [140]. If patients experienced multiple rejection episodes, the most severe rejection was reported. P values were calculated with the log-rank test. A diagnosis of ABMR required positive immunostaining for C4d, combined with either glomerulitis (g 1) or glomerulopathy (cg 1).

Table 3. Maintenance immunosuppression*

Variable	All patients		p value†	Immunologically low-risk patients		Immunologically high-risk patients		p value‡
	Rituximab (n = 138)	Placebo (n = 142)		Rituximab (n = 84)	Placebo (n = 81)	Rituximab (n = 20)	Placebo (n = 26)	
At three months after transplantation								
Trough levels of tacrolimus – ng/ml	8.3 ± 2.7	8.4 ± 2.1	0.78	8.0 ± 2.5	8.4 ± 2.1	9.2 ± 3.2	8.1 ± 2.1	0.21
Mycophenolate mofetil dose – mg/day	1500 (0 – 2000)	1500 (0 – 2500)	0.081	1500 (0 – 2000)	1500 (0 – 2500)	1500 (0 – 2000)	1500 (0 – 2000)	0.19
Steroid dose – mg/day	11.7 ± 3.3	11.7 ± 4.1	1.0	11.7 ± 3.3	11.9 ± 4.2	11.8 ± 3.5	11.1 ± 4.1	0.79
At six months after transplantation								
Trough levels of tacrolimus – ng/ml	7.4 ± 1.7	8.0 ± 2.2	0.047	7.4 ± 1.7	7.9 ± 2.4	7.5 ± 1.6	8.1 ± 1.6	0.25
Mycophenolate mofetil dose – mg/day	1500 (0 – 2000)	1500 (0 – 2500)	0.037	1500 (0 – 2000)	1500 (0 – 2500)	1500 (0 – 2000)	1500 (500 – 2000)	0.18
Steroid dose – mg/day	10.4 ± 5.0	9.3 ± 3.0	0.029	10.0 ± 3.5	9.5 ± 3.3	11.9 ± 8.5	8.5 ± 1.4	0.013

* Values are presented as mean ± standard deviation or median (range). Data are shown for patients on tacrolimus, mycophenolate mofetil and steroids.

† Calculated with the T test, except for the mycophenolate mofetil dose for which the Mann-Whitney U test was used.

‡ Calculated with the one-way ANOVA test, except for the mycophenolate mofetil dose for which the Kruskal-Wallis test was used.

Table 4. Secondary end points*

Variable	Rituximab (n = 138)	Placebo (n = 142)
Delayed graft function at six months (%)	18.8	17.6
Biopsy-proven acute rejection at 24 months (no. – %)	27 (19.6)	31 (21.8)
Calculated GFR at six months in patients with a functioning graft – ml/min†	51.3 ± 16.9	50.6 ± 17.0
Proteinuria at six months – g/10 mmol creatinine	0.16 (0.10 – 6.5)	0.18 (0.10 – 6.7)
Calculated GFR at 24 months in patients with a functioning graft – ml/min†	49.6 ± 16.2	51.6 ± 16.6
Proteinuria at 24 months – g/10 mmol creatinine	0.10 (0.10 – 10.6)	0.10 (0.10 – 3.5)
Allograft survival at six months (%)		
Censored for death of patients with functioning graft	96.4	93.0
Uncensored for death of patients with functioning graft	94.9	90.1
Allograft survival at end of follow-up (%)		
Censored for death of patients with functioning graft	92.0	87.3
Uncensored for death of patients with functioning graft	79.7	78.2
Patient survival at six months (%)	97.8	95.8
Patient survival at end of follow-up (%)	87.0	85.9
Cause of death (no.)	*number of patients*	
Infection related	7	5
Malignancy related	4	3
Other causes	5	7
Patients with 1 infection within six months (%)	63.8	62.0
Patients with 1 bacterial infection within six months (%)	33.3	34.5
Patients with 1 fungal infection within six months (%)	16.7	19.7
Patients with CMV disease within six months (%)	14.5	11.3

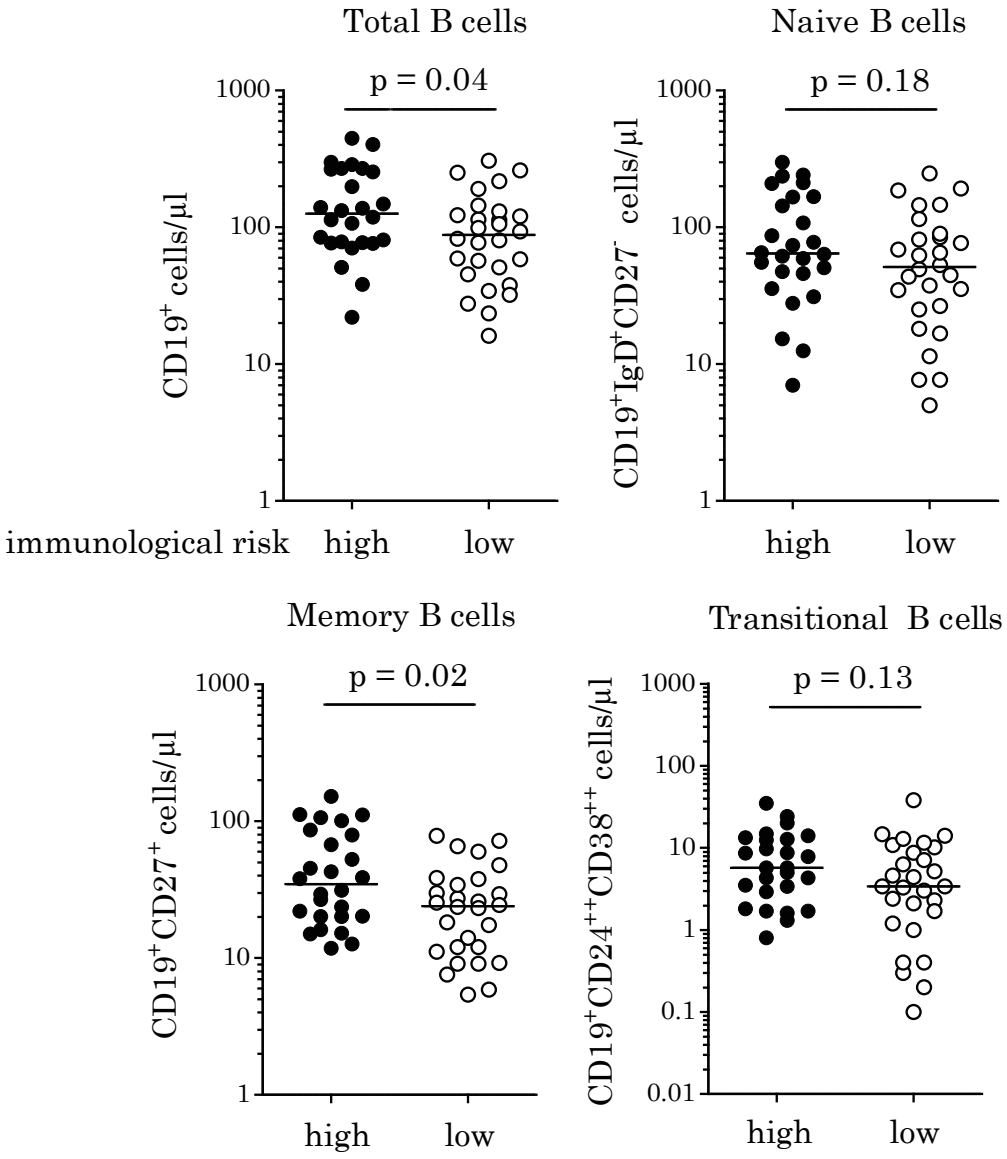
Patients with 1 infection within 24 months (%)	79.0	76.1
Patients with 1 bacterial infection within 24 months (%)	50.0	49,3
Patients with 1 fungal infection within 24 months (%)	22.4	25.4
Patients with CMV disease within 24 months (%)	15.9	12.7
Patients with 1 malignancy within 24 months (%)	5.8	5.6
Type of malignancies (no.)	*number of patients*	
Skin	2	5
Non-skin	6	3
Patients with 1 serious adverse event (SAE) within 24 months (%)	70.3	64.8
Incidence of grade 2 leucopenia within six months (leucocytes < 3.0 10^9/L) (%)‡	19.0	1.4
Incidence of grade 2 neutropenia within six months (neutrophils < 1.5 10^9/L) (%) ‡	24.3	2.2
Hemoglobin level at six months (mmol/L)	7.6 ± 1.0	7.6 ± 1.0
Thrombocyte count at six months (x10^9/L)	220 ± 69	222 ± 72

* Values are presented as mean ± standard deviation or median (range). For patients who have not reached follow-up of 24 months, data available until the last visit were used. Except for the incidence of leucopenia/neutropenia, group differences were not statistically significant as calculated with the T test, except for the number of serious adverse events and proteinuria, for which the Mann-Whitney U test was used.

† For the calculated GFR on the basis of abbreviated Modification of Diet in Renal Disease criteria, the following formula was used: estimated GFR (ml/min/1.73 m^2) = 175 (serum creatinine/88.4)$^{-1.154}$ (Age)$^{-0.203}$ (0.742 if female) (1.212 if African American) [142].

‡ $p < 0.001$ calculated with the Fisher's exact test.

Figure 3. Pre-transplant levels of B cells in immunologically high- vs. low-risk patients.*



* Absolute numbers (with medians) of total CD19+, naive (CD19+IgD+CD27-) and memory (CD19+CD27+) and transitional (CD19+CD24++CD38++) B cells in pre- transplantation blood samples of 26 immunologically high-risk and 28 matched immunologically low-risk patients. P values are calculated with the Mann-Whitney U test.

Discussion

Our data show that induction therapy with a single dose of rituximab at the time of renal transplantation is safe but ineffective to reduce the incidence of BPAR in a broad population of renal transplant patients. The pre-specified stratification of our patients according to retransplantation and PRA, enabled comparison of immunologically high and low-risk patients. We observed that within the whole population, immunologically high-risk patients who did not receive rituximab had the highest incidence of BPAR. A separate analysis on the subpopulation of immunologically high-risk patients showed a clear trend towards a lower incidence of BPAR with rituximab therapy as compared to placebo. Notably, the study was not sufficiently powered for this analysis. Interestingly, the incidence of ABMR tended to be lower after rituximab, especially in immunologically high-risk patients. Furthermore, within the immunologically high-risk subgroup, the incidence of delayed graft function tended to be lower in rituximab-treated patients, which could have contributed to a lower rate of rejection. Altogether, these results suggest a protective effect of rituximab against acute rejection in patients who are at higher immunological risk. With the current median duration of follow-up of 4.0 years, this beneficial effect has not resulted in improved graft function or graft survival.

Two recent randomized studies have reported on the effect of rituximab as induction therapy. In the first study, with 140 patients, Tydén et al. also showed no significant effect of rituximab on the incidence of BPAR within six months after transplantation, although a tendency toward fewer and milder rejection episodes in the rituximab group was observed (11.8% vs. 17.6%, $p = 0.32$) [106]. In the current study, we included twice as many patients and stratified for immunological risk. The other study by Clatworthy et al. was prematurely terminated after the inclusion of 13 patients because of an excess incidence of acute rejection in rituximab-treated patients [107]. In this study however, two doses of rituximab were given and maintenance immunosuppression was steroid-free. As possible explanation for their findings it was suggested that proinflammatory cytokine release associated with B cell depletion might prime antigen-presenting cells.

It is tempting to speculate that a protective effect of rituximab in immunologically high-risk patients could be explained by reducing the relatively high pre-transplant levels of memory B cells in our immunologically high-risk patients. Indeed, an increase in circulating memory B cells has been associated with acute rejection in pediatric renal transplant recipients, while heart transplant recipients with higher percentages of naive B cells had a lower risk of acute rejection [143, 144]. However, we and others have

previously shown that memory B cells are more resistant to depletion by rituximab than naive B cells [145, 146].

We have also noticed that in vitro rituximab can affect B cell phenotype and its antigen presenting function, resulting in an altered outcome of B-T cell interaction [147]. After in vivo treatment with one dose of rituximab, we found that a B cell population remains in secondary lymphoid organs, despite complete depletion in peripheral blood [146]. These remaining B cells mainly consist of switched memory (IgD-CD27+) B cells, and have different functional capacities as compared to B cells obtained from lymph nodes of patients not treated with rituximab. Taken together, these data indicate that rituximab can have effects beyond pure B cell depletion, but in general these effects appear to be insufficient to reduce the incidence of BPAR after renal transplantation.

Although rituximab does not directly target the antibody producing plasma cells, prolonged B cell depletion might lead to a decrease in the concentration of donor-specific anti-HLA antibodies. A recent study shows that elimination of peripheral HLA-specific B cells of sensitized patients prevented an amnestic antibody response posttransplant [148]. The routine measurement of these antibodies was not part of standard clinical care at the time of initiation of the current study, and not included in the trial design. Another drawback of our study was that protocol biopsies were not performed. Together with the routine measurement of donor-specific anti-HLA antibodies this could have provided additional information on the risk of development of chronic ABMR and subsequent future graft loss [149].

Our study confirmed previous observations of a high incidence of leucopenia and neutropenia after treatment with rituximab [112]. The cause of this so called late-onset neutropenia remains incompletely understood. The higher incidence of neutropenia did not lead to more infections, which is in accordance with the results of other studies [97, 150, 151]. Potentially worrisome are the 3-year follow-up data from Tydéns study, suggesting an increased mortality in rituximab-treated patients [152]. We could not confirm these results in our study, and overall, the addition of rituximab to a combination of tacrolimus, mycophenolate mofetil and steroids appeared to be safe. Nonetheless, these data, together with the study of Clatworthy, underline that B cell depletion must be undertaken with caution.

During the course of this clinical trial, the use of interleukin-2 receptor antagonists and depleting anti-T cell antibodies as induction therapy has become more common and is recommended in the KDIGO guidelines from 2009. The safety of combining rituximab

with these agents needs to be established formally, although in a retrospective analysis and uncontrolled cohort study the combination of pretransplant rituximab, as part of desensitization therapy, and posttransplant induction therapy with anti-T cell agents appeared to be safe [153, 154].

In conclusion, our randomized and placebo-controlled study has demonstrated that addition of rituximab induction therapy to a triple drug immunosuppressive regimen does not reduce the incidence of BPAR in immunologically low-risk patients. Therefore, we do not recommend the use of rituximab induction therapy in a population of unselected renal transplant recipients. However, our data suggest that treatment with rituximab may reduce the incidence of BPAR in immunologically high-risk patients to a level comparable to that in immunologically low-risk patients, thereby indicating a potential direction to improve the treatment of this specific group of patients. The results need to be confirmed in a multicenter clinical study focused on immunologically high-risk patients, with special attention for the combination or comparison with other induction agents, like basiliximab or anti-thymocyte globulin.

Acknowledgements

We thank Judith Kal-van Gestel for monitoring the data collection and analysis throughout the study. The study was performed at the nephrology unit of the Radboud University Medical Center and we acknowledge the contribution of all physicians and nurses involved in the study. We thank the participating patients for their confidence and contribution. We acknowledge Jan van den Brand for contribution to the statistical analysis. Preliminary data of this trial was presented at the American Transplant Congress 2013 in Seattle.

Chapter 7

Intragraft B cells during renal allograft rejection: effect of rituximab induction therapy

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Submitted

Abstract

The pathophysiological role of intragraft B cells during renal allograft rejection is unclear. We studied B cell infiltration during acute rejection in 30 patients who participated in a clinical trial in which adult renal transplant patients were randomized between a single dose of rituximab (375 mg/m^2) or placebo as induction therapy. Biopsies were examined independently by two blinded pathologists according to the Banff classification and scored for the presence of B cells and plasma cells using CD79a and CD138 as markers. The majority of acute rejections was T cell mediated. The proportion of acute rejections with an antibody mediated component did not differ between rituximab-treated patients (2/11, 18.2%) and placebo-treated patients (6/19, 31.6%; P=0.67), and there were also no differences in t-, v-, i-, or g-scores of the Banff classification. However, biopsies of rituximab-treated patients had a significantly lower B cell score (0.08, range 0.00 – 0.50) than placebo-treated patients (2.00, range 0.70 – 3.30, P<0.001). There was no difference in the plasma cell score. Depletion of intragraft B cells during acute rejection did not affect steroid-resistance, proteinuria, graft function, or patient- and graft survival at 4 years. In conclusion, these data do not support a pathogenic role for intragraft B cells during acute allograft rejection.

Introduction

Rituximab is a monoclonal antibody against the CD20 antigen present on different types of B cells. It is currently used in renal transplantation in different settings [155]. After administration of a single dose it quickly induces complete and long-lasting B cell depletion in the peripheral blood [110]. However, in secondary lymphoid organs, like lymph nodes and spleen, B cell depletion after a single dose of rituximab is incomplete [145, 146].

During acute allograft rejection, different cell types can infiltrate the graft, such as T cells, NK cells, monocytes, and also B cells. The clinical relevance of infiltrating B cells is a matter of debate. Some studies show an association with a poorer response to anti-rejection therapy and hence worse graft outcome, while other studies do not show a negative impact of B cell infiltration on graft outcome [101, 156-158]. Many case series have suggested a beneficial effect of rituximab in the treatment of (antibody mediated) renal allograft rejection, but these results are difficult to interpret without a control group. In a small randomized trial (n=20), treatment with rituximab (next to anti-thymocyte globulin and/or high-dose steroids) resulted in a larger improvement of graft function and of biopsy rejection scores at six months post-treatment, compared to treatment with anti thymocyte globulin and/or high dose steroids alone [151]. This improvement was accompanied by complete intragraft B cell depletion in all rituximab-treated patients at follow-up. Although rituximab had no apparent effect on donor specific antibody levels, reappearance of C4d deposition was not seen on follow-up biopsies after rituximab treatment. These findings suggest a pathogenic role of intragraft B cells in acute renal allograft rejection.

Next to its use for the treatment of rejection, rituximab has been studied in the setting of prevention of acute rejection. We have recently performed a double-blind, placebo-controlled study, in which 280 adult renal transplant patients were randomized between a single dose of rituximab (375 mg/m^2) or placebo during transplant surgery [159]. Patients were stratified according to panel reactive antibody (PRA) value and rank number of transplantation. Maintenance immunosuppression consisted of tacrolimus, mycophenolate mofetil, and steroids. This study showed that a single dose of rituximab as induction therapy did not reduce the overall incidence of biopsy proven acute rejection, but might be beneficial in immunologically high-risk patients.

Here we report the effect of rituximab on graft histology during acute rejection, with emphasis on the type of rejection (T cell mediated versus antibody mediated) and the

intragraft presence or absence of B cells. Important research questions were whether the presence of B cells was associated with a higher extent of steroid-resistance and worse graft function after follow-up and whether this was influenced by rituximab.

Patients and methods

The full details of the original clinical trial have been described elsewhere [159]. In brief, 280 patients were randomized to treatment with a single dose of 375 mg/m^2 body surface area rituximab (n=138) or placebo (n=142) added to standard immunosuppression consisting of tacrolimus, mycophenolate mofetil, and steroids. In this trial, biopsies were only performed on clinical indication. First line treatment of acute rejections consisted of methylprednisolone, followed by anti-T cell antibodies in case of steroid-resistance.

We selected all patients who had a biopsy proven acute rejection within six months post-transplantation. To rule out an effect of earlier antirejection treatment on graft histology, we only analyzed the first biopsy in each patient and excluded patients who received anti-rejection treatment with steroids for more than 1 day preceding the biopsy.

The biopsy material was bouin-fixated and four-micrometer sections were processed for routine histologic stains including hematoxylin-eosin, Jones' silver stain, Masson Trichrome, and periodic acidic Schiff after diastase treatment. Staining for C4d was performed on frozen sections using immunofluorescence technique, with a mouse polyclonal anti-human C4d antibody (Biogenesis Inc., Ede, The Netherlands). Four-micrometer sections were incubated with monoclonal antibodies directed at the B cell marker CD79a (M7050, clone JCB117 by Dako, Glostrup, Denmark) and plasma cell marker CD138 (ILM3825-c1 – clone B-A38 by Immunologic, Klinipath, Duiven, The Netherlands). As secondary antibody we used powervision Poly-HRP-anti Mouse/Rabbit/Rat IgG (Immunologic, Klinipath, Duiven, The Netherlands). Detection was carried out with the use of peroxidase as label and diaminobenzidine as substrate. We did not stain for CD20, since that staining could be falsely negative due to blockage of CD20 by rituximab. Moreover, we also did not use CD19 as a B cell marker, because a monoclonal antibody against CD19 for use in bouin-fixated tissue is not available. Since CD79a is also expressed on plasma cells we performed a immunohistochemical staining for CD138 to differentiate between B cells and plasma cells.

Biopsies were scored in a blinded fashion by two independent pathologists. Rejection was scored according to the Banff 07 criteria for T cell mediated rejection (TCMR) and

antibody mediated rejection (ABMR) [140]. According to the Banff 07 classification, diffuse C4d staining (i.e. >50% of peritubular capillaries) was defined as positive. To differentiate between clusters and scattered positive cells, CD79a+ cells were scored in a manner as previously described (for CD20+ and CD3+ cells) and CD138+ cells were scored as the number of positive cells per high power field [158]. Per biopsy specimen the whole cortex was examined. A cluster of cells was defined as more than 30 immunohistochemically positive cells without the interposition of tubules. For each high-power field, the scattered CD79a+ cells were scored according to an ordinal scale ranging from 0 to 5, as defined in Table 1. In each biopsy specimen, the total scores for CD79a+ cells, and the total numbers of CD79a+ clusters were divided by the number of high-power fields that were examined.

Statistical testing was performed according to distribution and type of data (unpaired T test, Mann-Whitney U test, chi-square test, or Fisher's exact test). Allograft loss and death were analyzed with the Kaplan–Meier method, and differences were assessed by the log-rank test. Correlation between histological scoring of CD79a and CD138 with clinical outcome was performed with a Spearman's rho correlation co-efficient. Analyses were performed with IBM SPSS Advanced Statistics 21.0 and GraphPad Prism 5.03.

Table 1. Histological scoring of interstitial CD79a+ cells

Histological scoring	No. positive cells per high power field
0	0 – 2
1	3 – 10
2	11 – 20
3	21 – 40
4	41 – 80
5	>80

Results

The clinical parent study included 280 renal transplant patients (138 rituximab-treated vs. 142 placebo-treated). Overall, the groups were well balanced with respect to demographic, clinical, and donor characteristics [159]. One or more renal biopsies were performed in 46 of the 138 rituximab-treated patients (33%) as compared to 60 of the 142 placebo-treated patients (42%, p=0.12 by chi-square test). In half of the cases (53/106) the biopsy showed acute rejection. Twenty-three cases were excluded because treatment with high dose steroids was started more than 1 day before the biopsy was taken. The remaining 30 patients (11 rituximab-treated and 19 placebo-treated) were

balanced with respect to demographic and clinical characteristics, but the number of living donors was substantially lower in the rituximab-treated patients (Table 2 and Figure 1). The median time from transplantation to rejection-biopsy was approximately 21 days (range 7 – 180 days). The results of the histological examination are shown in Table 3 and Figure 2. With respect to the type of rejection, ABMR and ABMR combined with TCMR was less frequent in rejection-biopsies from patients treated with rituximab, but this was not statistically significant (18.2% versus 31.6% p=0.67 by Fisher's exact test). No differences between groups were seen in other rejection-related items of the Banff scheme such as tubulitis, (t-score), arteritis (v-score), inflammation (i-score), and glomerulitis (g-score) (Figure 2).

Figure 1. Trial profile of all patients.

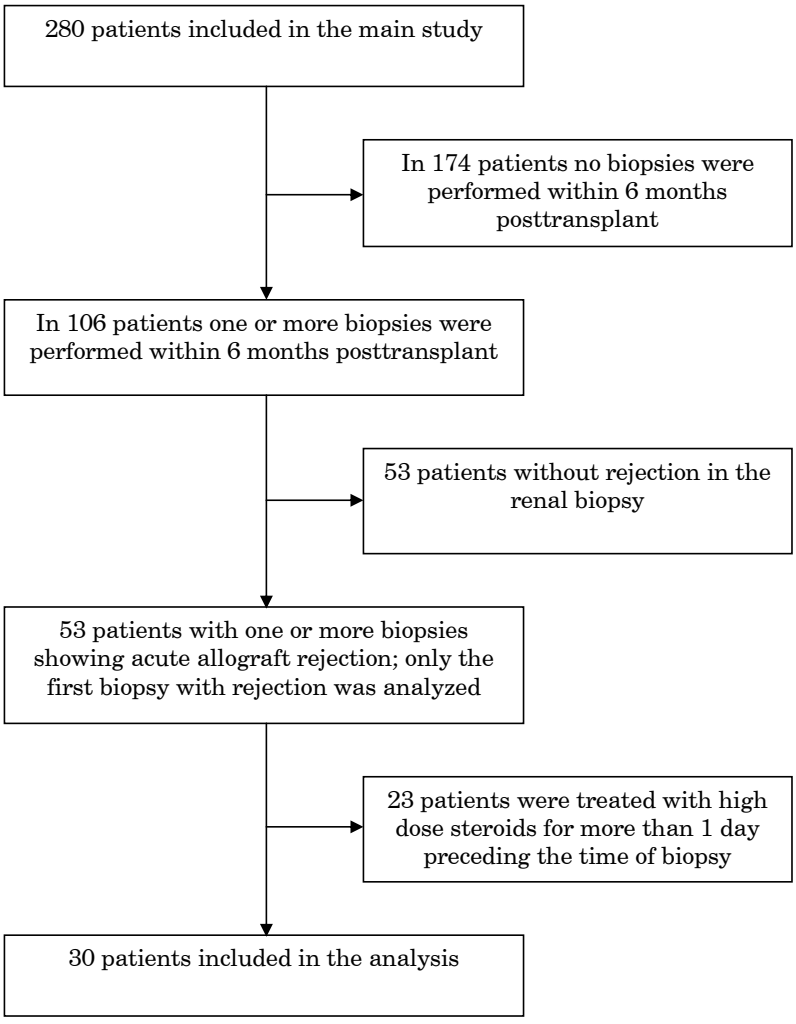

Figure 2. **Histological scores of biopsies during acute allograft rejection**

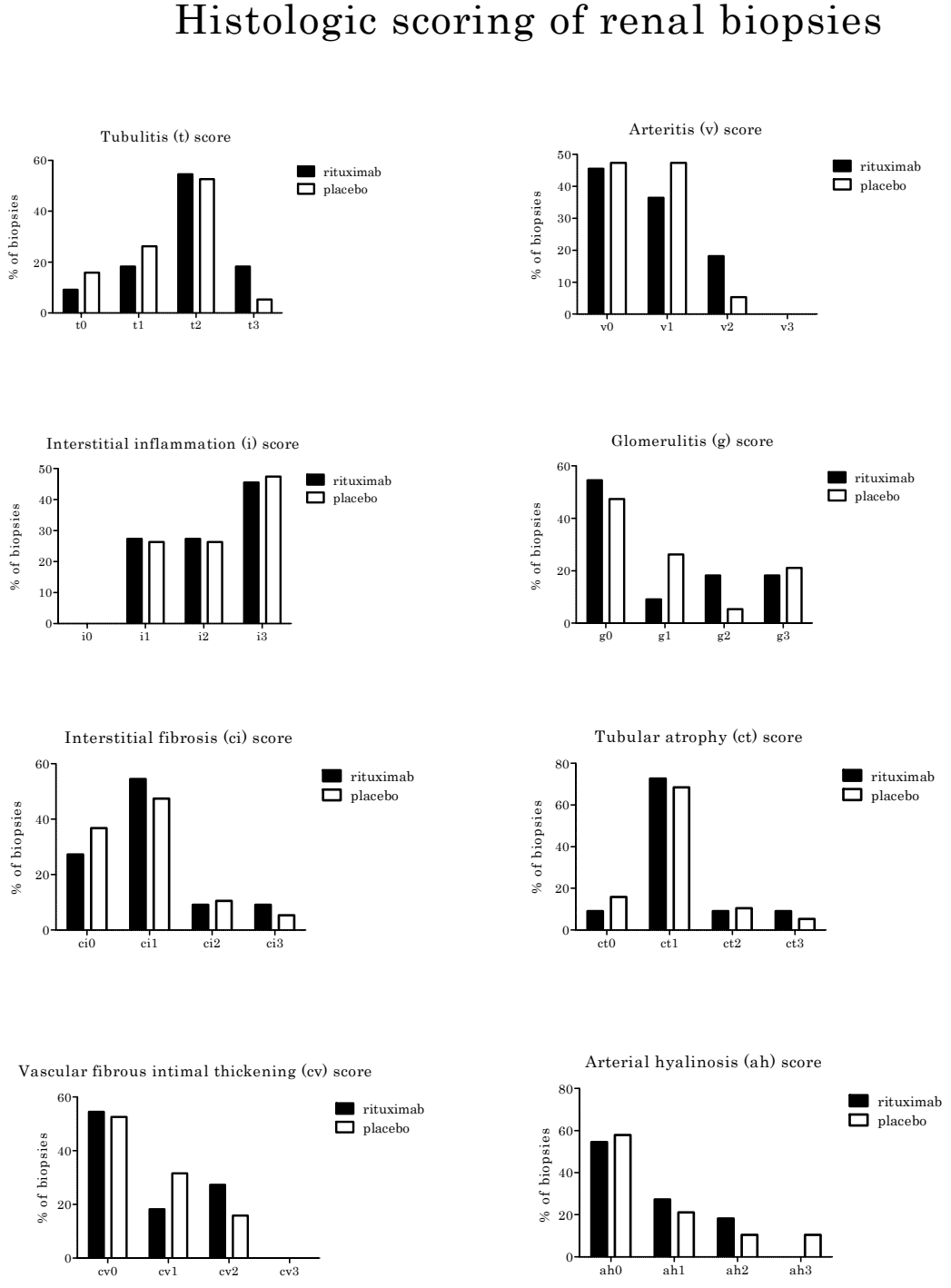

Table 2. Baseline characteristics of renal transplant recipients, having received either rituximab or placebo induction therapy, with biopsy-proven acute rejection *

Variable	Rituximab (n=11)	Placebo (n=19)
Age (yr)	48.8 ± 10.6	50.4 ± 8.2
Male sex (%)	54.5	47.4
White race (%)†	90.9	94.7
Cause of end-stage renal disease (no. of patients)		
Glomerulonephritis	2	10
Diabetes mellitus	1	1
Urological disorder	1	3
Hypertension / vascular damage	2	0
Polycystic kidney disease	3	3
Uncertain or other	2	2
Type of donor (%)		
Living	27.3	57.9
Deceased – donation after circulatory death	9.1	15.8
Deceased – donation after brain death	63.6	26.3
Donor age (yr)	53.4 ± 7.7	51.7 ± 9.1
HLA mismatches — A, B, and DR (no.)	2.73 ± 1.10	3.42 ± 1.12
Panel-reactive antibody titer — highest assessment	3 (0 – 42)	2 (0 – 47)
Patients with re-transplant (%)	0.0	15.8
Cold-ischemia time — deceased donors only (hr)	15.3 ± 4.2	16.0 ± 4.9
Days between transplantation and rejection-biopsy (days)	20 (7 – 180)	21 (7 – 138)
Estimated GFR at time of rejection – ml/min§	23 (0 – 35)	18 (0 – 48)

* Values are presented as mean ± standard deviation or median (range). Overall group differences were not significant.

† Race was determined by the investigator.

§ For the estimated GFR on the basis of abbreviated Modification of Diet in Renal Disease criteria, the following formula was used: estimated GFR (ml/min/1.73 m^2) = 175 x (serum creatinine/88.4)$^{-1.154}$ x (Age)$^{-0.203}$ x (0.742 if female) (1.212 if African American). In case of continued dialysis treatment a GFR of 0 ml/min was used [142].

Remarkably, the score for interstitial CD79a+ cells was significantly lower in rituximab-treated patients (median 0.08, range 0.00 – 0.50), compared to placebo-treated patients (median 2.00, range 0.70 – 3.30, P<0.001 by Mann-Whitney U test, Figure 3). Clusters of CD79a+ cells were found in the biopsies of four placebo-treated patients while these were never present in biopsies of rituximab-treated patients. Of the four placebo-treated

patients with CD79a+ cell clusters in their biopsies, three had functioning grafts at the end of follow-up, while one patient died after three years with a functioning graft. No difference was found in CD138 staining of biopsies between rituximab-treated patients (median 0.15, range 0 – 1.90) and placebo-treated patients (median 0.4, range 0 – 7.50; P = 0.45 by Mann-Whitney U test, Figure 3). Clusters of CD138+ were found in the biopsies of two placebo-treated patients, and not in biopsies of rituximab-treated patients. None of the biopsies showed evidence of the formation of ectopic lymphoid tissue (tertiary lymphoid organs).

Figure 3. CD79a+ and CD138+ scores in renal biopsies during acute allograft rejection

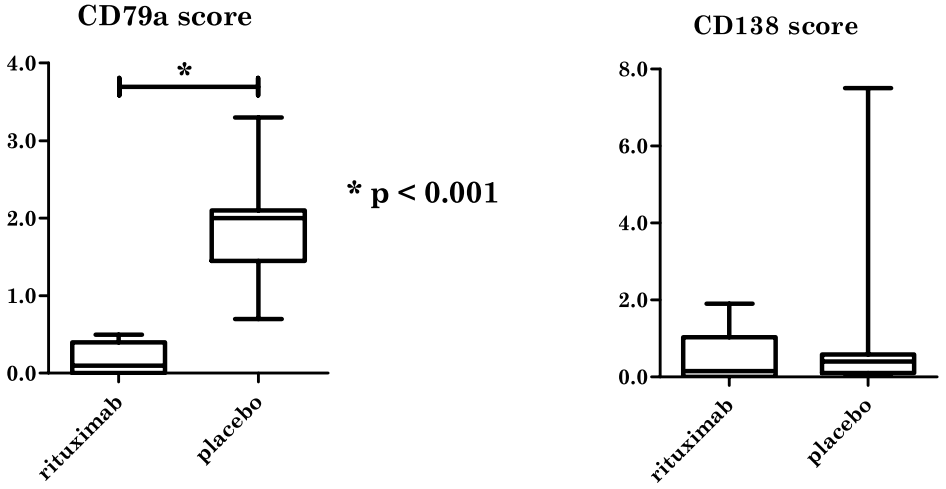

Table 3. Incidence and type of biopsy proven acute rejection (BPAR) at six months*

Variable	Rituximab (n=11)	Placebo (n=19)
T cell mediated rejection (no.)		
Type IA	4	4
Type IB	1	1
Type IIA	3	7
Type IIB	1	1
Antibody mediated rejection (ABMR; no.)	0	1
Combined rejections (no.)		
ABMR + Type IIA	1	5
ABMR + Type IIB	1	0
C4d positive (no – %)	2 (18.2)	6 (31.6)

* Biopsies were independently scored by two pathologists according to the Banff 07 classification [140]. A diagnosis of ABMR required positive immunostaining for C4d, combined with either signs of microvascular inflammation (g>0 and/or ptc>0) or intimal arteritis (v>0)

Although induction with a single dose of rituximab led to an almost complete absence of intragraft CD79a+ cells, this did not translate into a beneficial effect on clinical outcomes in these patients with biopsy-proven acute rejection. The percentage of steroid-resistant rejections did not differ between rituximab and placebo-treated patients (36.4% versus 47.4%, P=0.71 by Fisher's exact test). Furthermore, there was no difference in proteinuria or graft function, at two years post-transplant. Patient- and graft survival were comparable in both groups (Table 4). In the placebo-treated patients, the biopsy score for interstitial CD79a+ cells was not correlated with either improvement of eGFR (from moment of rejection to 24 months post-transplant; Spearman's correlation co-efficient -0.007, P=0.98) or absolute eGFR at 24 months post-transplant (Spearman's correlation co-efficient 0.20, P=0.48). Interestingly, in placebo-treated patients the score for interstitial CD79a+ cells was significantly lower in patients with ABMR (median 1.25, range 0.70 – 2.00) than in patients without ABMR (median 2.00, range 1.40 – 3.30, P=0.005 by Mann-Whitney U test). In placebo-treated patients, neither the presence of clusters of CD79a+ cells nor of CD138+ cells showed a significant correlation to clinical outcome.

Table 4. Outcomes after transplantation in patients with biopsy-proven acute rejection*

Variable	Rituximab (n=11)	Placebo (n=19)
Patients with steroid-resistant rejection (no – %)	4 (36.4)	9 (47.4)
Estimated GFR at two years in patients with a functioning graft – ml/min†	34 (31 – 49)	38 (16 – 64)
Improvement of GFR from time of rejection till two years post-transplant in patients with a functioning graft at two years – ml/min	11 (7 – 35)	14 (-5 – 55)
Proteinuria at two years – g/10 mmol creatinine	0.10 (0.1 – 0.8)	0.10 (0.1 – 4.0)
Allograft survival at end of follow-up (%)‡		
Censored for death of patients with functioning graft	81.8	89.5
Uncensored for death of patients with functioning graft	63.6	78.9
Patient survival at end of follow-up (%)‡	72.7	84.8

* Values are presented as mean ± standard deviation or median (range).

† For the estimated GFR on the basis of abbreviated Modification of Diet in Renal Disease criteria, the following formula was used: estimated GFR (ml/min/1.73 m^2) = 175 x (serum creatinine/88.4)$^{-1.154}$ x (Age)$^{-0.203}$ x (0.742 if female) (1.212 if African American) [142].

‡ median duration of follow-up of 4.1 years, range 2.0–6.2 years.

Discussion

Infiltration of B cells is a frequent finding in biopsies of patients with acute renal allograft rejection. Our data show that in renal transplant patients who received a single dose of rituximab as induction therapy, intragraft B cells were nearly absent during episodes of acute allograft rejection. Moreover, the relative frequency of pure ABMR or combined ABMR and TCMR was lower than in placebo-treated patients. No differences were seen in the severity of tubulitis, arteritis, or the extent of the cellular infiltrate.

We and others previously showed that a single dose of rituximab at time of renal transplant surgery results in a rapid and long lasting depletion of B cells in the peripheral blood [110, 160]. Even at two years after transplantation, the absolute number of B cells in peripheral blood was still quite low as compared to patients not treated with rituximab [160]. We also showed that despite complete depletion of B cells in the peripheral blood the number of B cells in secondary lymphoid organs remained unaffected [146]. Others have confirmed that in the near absence of B cells in peripheral blood there is a varying degree of reduction, but no complete depletion, of B cells in

spleen, lymph nodes, and synovial tissue after treatment with rituximab [110, 161-163]. Together with our current findings, these data suggest that rituximab inhibits the egress of B cells from secondary lymphoid organs, thereby impairing the migration to peripheral tissues such as the renal allograft.

Earlier studies by Hippen et al. [101] and Sarwal et al. [3], demonstrated that the presence of CD20+ B cell infiltrates in the graft at time of rejection is a bad prognostic sign. It was suggested that this could be explained by the antigen presenting function of infiltrating B cells. Based on these findings, it could be expected that the absence of intragraft B cells in rituximab-treated patients had resulted in a lower rate of steroid-resistance and better graft survival. However, our data do not indicate that absence of intragraft B cells during acute rejection, as observed in rituximab-treated patients, translates into better outcome. A potential bias hampering the interpretation of this finding could have been a selection of more severe rejections occurring despite B cell depletion in the rituximab group. This was unlikely however, since Banff scores for tubulitis, arteritis, inflammation, and glomerulitiswere similar in biopsies of rituximab-treated and placebo-treated patients. Also within the placebo-treated patients there was no correlation between the diffuse and scattered or clustered presence of B cells on the one hand and the clinical course on the other hand. In four placebo-treated patients B cell clusters were found, and after a median follow-up of 4 years three of the four patients still had a good and stable graft function. Notably, other authors also could not demonstrate that intragraft B cells were associated with a poorer response to high dose steroids or worse graft survival [157, 158, 164-166].

In the parent clinical trial overarching this study, a clear trend towards less ABMR was seen in rituximab-treated patients, compared to placebo-treated patients (4/138, 2.9% vs. 11/142, 7.7% $p = 0.11$ by Fisher's exact test). The near complete depletion of intragraft B cells could have been the mechanism responsible for this finding. However, as stated above, data about B cells in other lymphoid compartments are essential for correct interpretation. This is underlined by the finding that placebo-treated patients with ABMR had lower scores for interstitial CD79a+ cells than placebo--treated patients without ABMR, which suggests that B cells and plasma cells which are involved in the process of ABMR are residing outside the graft. Taken together, the findings in our study question the pathologic role of intragraft B cells in the acute phase of renal allograft rejection. Yet, it remains unclear why intragraft B cells are present in some circumstances, but not in others.

To conclude, induction therapy with rituximab strongly reduced the number of infiltrating B cells during acute renal allograft rejection. This did not have an effect on the severity of tubulitis, arteritis or the extent of the cellular infiltrate, nor did it improve clinical outcome after treatment of the rejection during a follow-up of 4 years. These data do not support previous findings suggesting that intragraft B cells which are present during acute allograft rejection are harmful to the graft.

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Chapter 8

Cytokine release after treatment with rituximab in renal transplant recipients

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Abstract

Background. Treatment with rituximab may be accompanied by a systemic cytokine release. We studied the effects of a single dose of rituximab on cytokine levels in transplant patients and examined the underlying mechanism.

Methods. Twenty renal transplant recipients (10 rituximab-treated, 10 placebo-treated) were recruited from a randomized clinical trial. Rituximab or placebo was infused during surgery and blood samples were taken before, during, and after surgery and analyzed for IL-2, IL-4, IL-6, IL-10, IL-12, IL-17, IFNγ, MIP-1ß, TGFß, and TNF . In vitro, healthy donor peripheral blood mononuclear cells, purified B cells, monocytes, NK cells, or combinations thereof were incubated with rituximab, rituximab-F(ab')$_2$, or medium and MIP-1ß, IL-10, IFNγ, and TNF levels were measured in the supernatant.

Results. Rituximab-treated patients had higher serum levels of IL-10 (101 ± 35 pg/ml vs. 41 ± 9 pg/ml; $p < 0.01$) and MIP-1ß (950 ± 418 pg/ml vs. 125 ± 32 pg/ml; $p < 0.001$) compared to placebo-treated patients at 2 hours after start of infusion. There was no difference in the level of other cytokines. In vitro, the addition of rituximab, but not rituximab-F(ab')$_2$ fragments, only led to significantly increased levels of MIP-1ß in co-cultures of B and NK cells. Levels of MIP-1ß were higher in patients with a high affinity Fc-receptor compared to those with a lower affinity FcγRIIIa (1356 ± 184 pg/ml vs. 679 ± 273 pg/ml; $p < 0.01$).

Conclusions. In addition to B cell depletion, rituximab can modulate the immune response by inducing cytokine secretion, especially IL-10 and MIP-1ß. Rituximab-induced MIP-1ß secretion depends on the combined presence of B cells and FcR-bearing cells, especially NK cells.

Introduction

Monoclonal antibodies are widely used in the treatment of malignancies, transplant rejections, as well as a range of autoimmune diseases. For several of these monoclonal antibody, administration has been associated with acute infusion reactions, caused by various mechanisms, including systemic inflammatory response syndrome [167]. Rituximab, a chimeric anti-CD20 monoclonal antibody, is an effective treatment for malignant lymphomas and various autoimmune diseases [168]. It is also used in organ transplant patients for desensitization and treatment of antibody-mediated rejection [105]. Rituximab can deplete B cells via antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and apoptosis [169]. The observation that polymorphisms in the FcγRIIIa gene affect the effectiveness of rituximab, indicates that ADCC plays an important role [170]. NK cells express the Fc-receptor FcγRIIIa (CD16) which has a high affinity for binding to IgG1, the isotype of rituximab [171]. FcγRIIIa appears in two allelic forms that differ by the amino acid on position 158. FcγRIIIa homozygous for valine (VV) has a higher affinity for IgG1 than FcγRIIIa with phenylalanine at that position (VF or FF) [172]. Patients with the high affinity receptor show a better clinical response to rituximab [173-175].

However, administration of rituximab can be followed by acute infusion reactions due to the release of cytokines, especially in patients with high B cell counts like lymphoma patients, although not every patient with elevated levels of cytokines develops clinical symptoms [167, 176-178]. After stimulation, B cells can produce virtually any cytokine [179]. Although the role of these cytokines in the regulation of other cell types is not completely understood, a rise in the level of (proinflammatory) cytokines could lead to activation of the immune system and therefore be of clinical relevance. When using rituximab as induction therapy to prevent acute rejection, a cytokine release could potentially contribute to the risk of rejection.

Currently, it is unknown to which extent cytokine release occurs in patients with normal B cell numbers, and the cell type responsible for the cytokine release has not been identified. In a double-blind placebo-controlled study, we measured the release of various cytokines after administration of rituximab to renal transplant recipients. In addition, we studied the mechanism of this cytokine release in vitro.

Materials and Methods

Patients

For this study, patients were recruited from the rituximab in Renal Transplantation trial (ClinicalTrials.gov, NCT00565331), which evaluated the efficacy and safety of rituximab (MabThera, Hoffmann-La Roche, Basel, Switzerland) when added to standard immunosuppression in renal transplant recipients. At the start of transplant surgery, patients received 1000 mg acetaminophen, 100 mg prednisolone and 2 mg clemastine i.v. next to the standard treatment with 2000 mg ceftriaxone as antibiotic prophylaxis. After 30 minutes, rituximab (375 mg/m^2) or placebo was administered at an increasing infusion rate. The total infusion time was approximately 4 hours.

For logistic reasons, only recipients of a living donor kidney were selected for this study. Peripheral blood samples were collected from 20 patients (10 rituximab-treated, 10 placebo-treated) a few hours before the transplantation (baseline), at 2 and 4 hours after starting the rituximab infusion (t = 2h and t = 4h), and the next morning (t = 24h). Sera were stored at -80˚C until analysis. **Table 1** summarizes the patient characteristics.

Table 1. Patient characteristics*

	Patient groups	
	Rituximab (n = 10)	Placebo (n = 10)
Age; mean ± SD	46.7 ± 12.5	44.7 ± 12.5
Sex; male : female, no.	6 : 4	9 : 1
HLA mismatches; mean ± SD	2.9 ± 1.3	3.3 ± 1.3
Immunosuppression before transplantation; no.	0	1
Patients with panel reactive antibody > 6%; no.	1	3
Renal replacement therapy		
none / hemodialysis / peritoneal dialysis; no.	3 / 6 / 1	2 / 7 / 1

* There were no statistical differences between the rituximab and placebo group.

Healthy donors and cell isolation

For in vitro experiments, peripheral blood samples were obtained from healthy donors after written informed consent. Peripheral blood mononuclear cells (PBMCs) were

isolated by density gradient centrifugation (Lymphoprep; Nycomed Pharma, Roskilde, Denmark). CD14+ Monocytes and CD19+ B cells were positively selected using specific-magnetic microbeads, and NK cells were negatively selected using the NK cell isolation kit II (all from Miltenyi Biotec, Bergisch Gladbach, Germany) resulting in a purity of more than 95% for all lymphocyte subsets.

Culture conditions

Whole blood culture was used to determine the cytokine production in vitro. Therefore, whole blood was diluted 1:5 with culture medium in 24-well plates (Greiner Bio-One, Frickenhausen, Germany). To study the cytokine production by different lymphocyte subsets, 2 10^5 PBMCs, B cells, NK cells, and/or monocytes were cultured in RPMI-1640 medium supplemented with pyruvate (0.02 mM), glutamax (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) (all from Gibco, Paisley, United Kingdom), and 10% fetal bovine serum (FBS) in 96-well round bottom plates (Greiner Bio-One) in a 37 C, 95% humidity, 5% CO2 incubator. In selected conditions, 250 µg/ml rituximab or 250 µg/ml rituximab-F(ab')$_2$ (provided by Genmab, Utrecht, The Netherlands) was added to the culture medium. Supernatant was collected after 14 hours and/or 24 hours and stored at -20˚C until analysis.

Cytokine measurements

Serum and culture supernatant levels of IL-2, IL-6, IL-10, IL-12, IL-17, IFNγ, TGFß, TNF (eBioscience, San Diego, CA), and MIP-1ß (Invitrogen, Carlsbad, CA) were determined by ELISA according to the manufacturer's instructions. IL-4 levels were determined by Luminex according to the manufacturer's instructions (Biorad, Veenendaal, The Netherlands).

FcγRIIIa-158 genotype analysis

DNA was extracted from peripheral blood of 10 rituximab-treated patients using a DNA isolation kit (Qiagen, Valencia, CA). Genotyping of FcγRIIIa-158 (rs396991) was performed using the TaqMan-Allelic discrimination method with a specific probe for rs396991 designed for single nucleotide polymorphism (SNP) of FcγRIIIa and results were analyzed using the Allelic Discrimination software program according to the manufacturer's instructions (all from Applied Biosystems, Foster City, CA).

Statistical analysis

Cytokine concentrations are presented as mean ± SD. Nonparametric tests were used to compare variables. One-way ANOVA was used to compare the different groups over time, followed by Dunn's multiple comparison test for post-testing. To test the difference between the cultured cells with rituximab or without (culture medium) the Wilcoxon signed rank test was performed. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad Prism 5.03 (GraphPad Software Inc., La Jolla, CA, USA).

Results

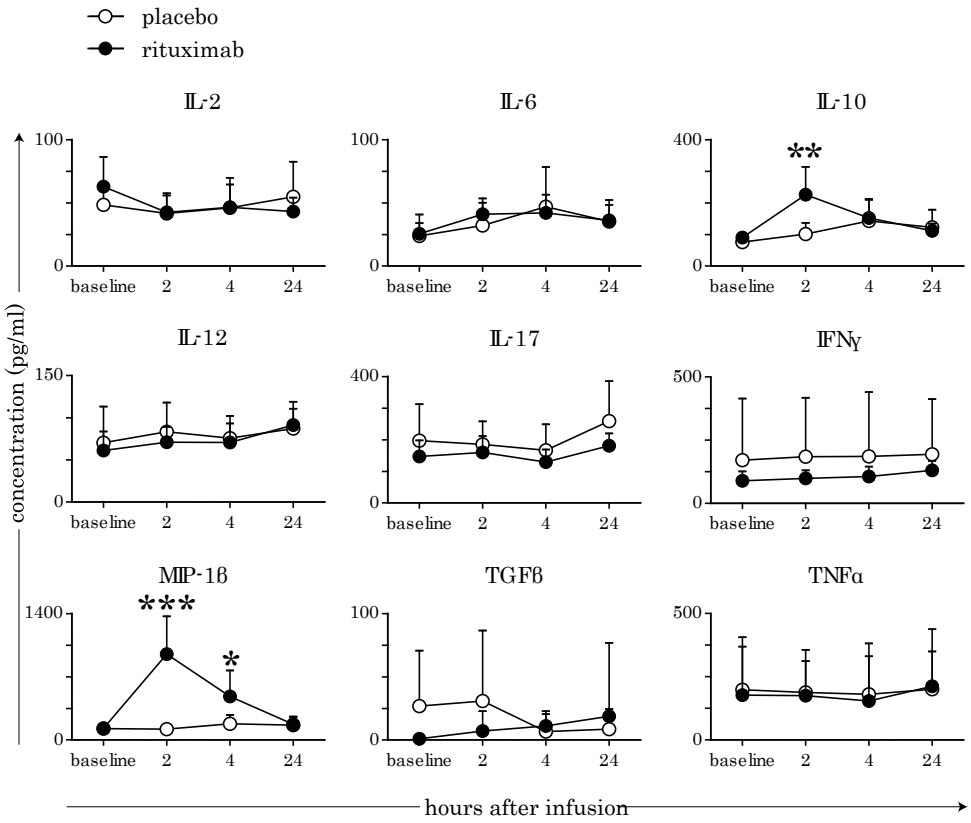
Rituximab infusion is associated with specific cytokine release

The infusion of rituximab induced a temporary rise in the levels of IL-10 and MIP-1ß. At 2 hours after the start of the infusion, the serum levels of these cytokines were significantly higher in rituximab-treated patients than in placebo-treated patients (IL-10 (101 ± 35 pg/ml vs. 41 ± 9 pg/ml; $p < 0.01$) and MIP-1ß (950 ± 418 pg/ml vs. 125 ± 32 pg/ml; $p < 0.001$; **Figure 1**). However, rituximab infusion did not increase the levels of IL-2, IL-4, IL-6, IL-12, IL-17, IFNγ, TGFß, or TNF levels as compared to placebo treatment, suggesting that rituximab infusion is associated with a specific cytokine release. Of note, the serum levels of IL-4 were below 1.2 pg/ml, which is the detection limit of the assay. None of the patients experienced any clinical symptoms associated with cytokine release.

Exposure to rituximab induces a B cell dependent secretion of MIP-1ß by NK cells

Using in vitro studies with blood from healthy donors, we next analyzed which cells were responsible for the cytokine production after exposure to rituximab. We used whole blood cultures next to PBMC, to stay as close to the in vivo situation as possible [180]. In a 24 hours whole blood culture as well as in isolated PBMC culture, addition of rituximab induced elevated MIP-1ß levels as compared to the medium alone (whole blood culture: 592 ± 218 pg/ml vs. 79 ± 68 pg/ml; $p = 0.06$ and PBMC culture: 500 ± 450 pg/ml vs. 23 ± 6 pg/ml; $p < 0.05$), whereas IL-10, IFNγ, and TNF were not detectable in this system (**Figure 2A**).

Figure 1. In vivo cytokine release after rituximab infusion.



Serum levels of IL-2, IL-6, IL-10, IL-12, IL-17, IFNγ, MIP-1ß, TGFß, and TNF in patients who underwent renal transplantation. 20 patients received a single dose of rituximab (375 mg/m^2) or placebo during transplant surgery. Concomitant immunosuppression consisted of tacrolimus, mycophenolate mofetil (MMF) and steroids. Blood samples were taken a few hours before transplantation (t = baseline), 2 and 4 hours after the start of infusion of rituximab or placebo, and the next morning after transplantation (t = 24 hours). Results are depicted as mean ± SD. Significant differences between placebo and rituximab-treated patients at the different time points are indicated by asterisks: **$p < 0.01$, ***$p < 0.001$.

To pinpoint the cells that produced MIP-1ß after rituximab administration, MIP-1ß levels were measured in the culture supernatant of freshly isolated PBMC, B cells, NK cells, or monocytes that were exposed to rituximab for 14 hours. In addition, MIP-1ß was measured in co-cultured B-NK cells and co-cultured B cell-monocytes upon rituximab treatment. rituximab did not increase the MIP-1ß levels in cultures of B cells, NK cells or

monocytes alone, nor in co-cultures of B cells-monocytes. In contrast, rituximab-treated co-cultures of B cells and NK cells revealed significantly enhanced MIP-1ß levels as compared to the medium control (597 ± 321 pg/ml vs. 30 ± 23 pg/ml; $p < 0.05$; **Figure 2B**). Intracellular MIP-1ß staining of the co-cultured B-NK cells showed that NK cells produce MIP-1ß upon exposure to rituximab for 14 hours, while B cells do not (**Figure 3**). Taken together, this suggests that after exposure to rituximab the observed increase in MIP-1ß is mainly due to secretion by NK cells in a B cell-dependent manner.

MIP-1ß secretion after exposure to rituximab is Fc-receptor dependent

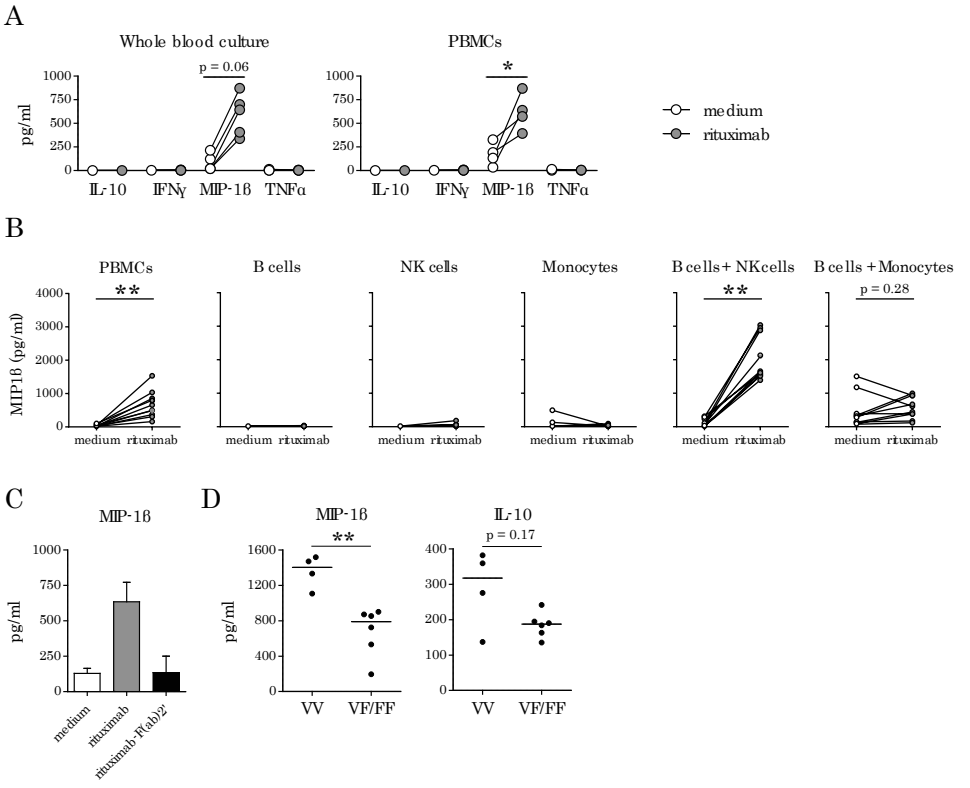
To establish if the MIP-1ß secretion caused by exposure to rituximab is Fc-receptor dependent, PBMCs were incubated with rituximab, rituximab-$F(ab')_2$ or in culture medium alone. PBMCs cultured with rituximab-$F(ab')_2$ did not show increased MIP-1ß secretion (**Figure 2C**), indicating that MIP-1ß secretion is Fc-receptor dependent.

It is known that single nucleotide polymorphisms (SNP) of the FcγRIIIa gene at position 158 result in an altered Fc-receptor function, where FcγRIIIa homozygous for valine (VV) has a higher affinity for IgG1 than FcγRIIIa with phenylalanine at that position (VF or FF) [172]. We therefore wondered whether the MIP-1ß secretion after treatment with rituximab in vivo could be correlated to the genotype of the Fc-receptor FcγRIIIa (CD16) present on NK cells. To this end, we determined the SNP at position 158 in 10 rituximab-treated renal transplant patients. Interestingly, rituximab-treated patients in the high affinity group (VV) revealed higher serum levels of MIP1ß as compared to the lower affinity group (VF/VV) at 2 hours after the start of the rituximab infusion (1356 ± 184 pg/ml vs. 679 ± 273 pg/ml; $p < 0.01$) (**Figure 2D**). Although not significant, a similar trend was observed for IL-10 levels (288 ± 111 pg/ml vs. 184 ± 35 pg/ml; $p = 0.17$). These findings support our conclusion from the in vitro studies that the cytokine release is Fc-receptor dependent.

Discussion

In this study, we showed that rituximab infusion leads to a specific cytokine release in renal transplant recipients. At 2 hours after the start of the infusion, IL-10 and MIP-1ß serum levels were significantly higher in rituximab-treated patients as compared to placebo-treated patients, whereas the levels of IL-2, IL-4, IL-6, IL-12, IL-17, IFNγ, TGFß, and TNF remained unaffected. Additional in vitro data revealed that NK cells were largely responsible for the MIP-1ß release in a B cell and Fc-receptor dependent manner.

Figure 2. Cytokine release after exposure to rituximab is Fc-receptor mediated.



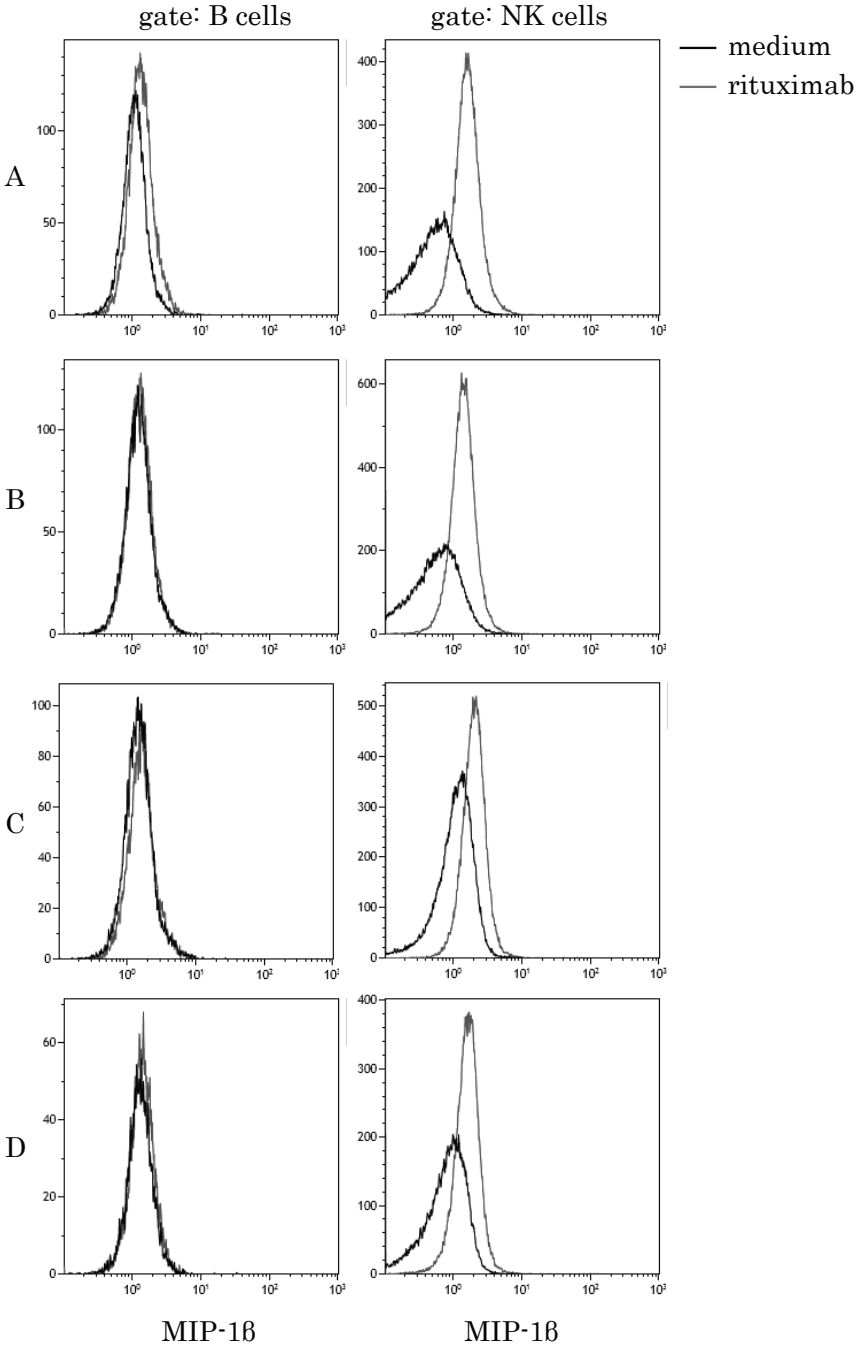
(A) Production of MIP-1β, IL-10, TNF and IFNγ from whole blood cultures (n = 5) and PBMCs of healthy donors (n = 4) incubated with medium or 250 µg/ml rituximab for 24 hours.

(B) Production of MIP-1β by PBMCs, isolated $CD19^+$ B cells, $CD3^-CD56^+$ NK cells, $CD14^+$ Monocytes, and a co-culture of B cells with NK cells or monocytes of healthy donors (n = 10) incubated with medium or 250 µg/ml rituximab after 14 hours.

(C) Production of MIP-1β by PBMCs of healthy donors 24 hours after incubation with medium, 250 µg/ml rituximab or rituximab-$F(ab')_2$.

(D) Serum levels of MIP-1β and IL-10 in patients with the high affinity receptor SNP-158VV (n = 4) and the low affinity receptor SNP-158VV/VF group (n = 6) 2 hours after the start of the rituximab infusion. Significant differences are indicated by asterisks: *$p < 0.05$, **$p < 0.01$.

Figure 3. Intracellular MIP-1β expression in co-cultures of B and NK cells of four different (A to D) healthy donors.



Although rituximab is usually well tolerated, the first infusion may be accompanied by severe side effects, which are correlated with complement activation and with the number of circulating B cells, as more side effects occur in patients with high B cell counts, like lymphoma patients [176, 181]. There is limited data on the cytokine releasing effects of rituximab in patients with B cell counts in the normal range, like renal transplant recipients or patients with autoimmune diseases. In our study cohort, we found elevated cytokine levels after rituximab infusion. The type of cytokines secreted was different from that observed in other cohorts treated with distinct monoclonal antibodies [171, 182]. The cytokine release syndrome associated with OKT3 treatment is characterized by the release of the inflammatory cytokines IL-2, TNF , and IFNγ, while treatment with the humanized anti-CD52 monoclonal antibody alemtuzumab was accompanied with elevated levels of IL-6, TNF , and IFNγ [182, 183]. In these cases, it is believed that the release of inflammatory cytokines is due to direct T cell activation or dependent on Fc-receptor ligation on phagocytic cells, such as monocytes, macrophages, and NK cells [182, 184]. We found that exposure to rituximab, without further stimulus, only led to significantly increased MIP-1ß levels in co-cultures of purified B and NK cells. Intracellular MIP-1ß staining of the co-cultured B-NK cells showed that NK cells produce MIP-1ß upon 14 hours exposure to rituximab, while B cells do not, suggesting that the cytokine release observed in vivo is coming from NK cells and largely dependent on the binding of these cells to rituximab-coated B cells. Upon recognition, NK cells can quickly release chemokines, such as MIP-1ß, whereas the release of TNF and IFNγ occurs hours later, which might explain the lack of TNF and IFNγ production in our in vitro cultures [185]. Finally, incubation of PBMCs with rituximab-F(ab')$_2$ did not lead to an increased MIP-1ß secretion, indicating that the cytokine release is indeed Fc-receptor dependent.

In patients with autoimmune diseases, several single nucleotide polymorphisms (SNPs) in Fcγ-receptors have been associated with the clinical response after treatment with monoclonal antibodies such as rituximab [170, 171, 186]. NK cells express the Fc-receptor FcγRIIIa (CD16) which has a high affinity for binding to IgG1, the isotype of rituximab [171]. FcγRIIIa appears in two allelic forms that differ by the amino acid on position 158. SNPs at position 158 result in an altered Fc-receptor function and have been associated with clinical response to rituximab [170, 187]. FcγRIIIa homozygous for valine (VV) has a higher affinity for IgG1 than FcγRIIIa with phenylalanine at that position (VF or FF) [172]. Interestingly, we found that MIP-1ß levels in patients treated with rituximab correlated to the FcγRIIIa-158 SNP. Patients with the high affinity SNP

produced higher levels of MIP-1ß. This observation fits with previous data showing that the degree of NK cell activation and the clinical response upon rituximab treatment are influenced by the FcγRIIIa-158 SNP [188].

The observed rise in IL-10 and MIP-1ß could be of clinical relevance, since it may well modulate the (allo)immune response influencing the transplantation outcome. IL-10 is a well-known anti-inflammatory cytokine which plays an important role in immune regulation [189]. In a mouse model, MIP-1ß was shown to be the most potent chemoattractant for regulatory T cells within a range of chemokines, suggesting that increased secretion might down modulate the immune response [190]. On the other hand, MIP-1ß can be produced by lymphocytes that are involved in inducing immune reactivity, including NK cells and B cells [191]. Moreover, MIP-1ß recruits monocytes, T cells, and dendritic cells to the site of injury or inflammation via the chemokine receptor CCR5, which is highly expressed by monocytes and has a lower expression on T cells and dendritic cells. Therefore, in renal transplantation increased MIP-1ß levels might contribute to activation of the immune response, and thus graft injury. Indeed, in a previous study with rituximab as induction therapy in renal transplantation, an increased rate of acute rejection was associated with elevated levels of proinflammatory cytokines in some of the patients one week after transplantation [107]. This suggests that rituximab might also have delayed effects on the cytokine production. In that report no data are provided on MIP-1ß levels.

In summary, results from this study indicate that rituximab does not only lead to B cell depletion, but also results in the release of cytokines, especially IL-10 and MIP-1ß, which might modulate the immune response. The MIP-1ß secretion appears to be B cell and Fc-receptor dependent.

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Chapter 9

The effects of in vivo B cell depleting therapy on ex vivo cytokine production

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Abstract

In renal transplantation, IL-17 production by T cells might be dependent on the presence of B cells. Therefore, the effect of in vivo B cell depletion on ex vivo IL-17 production was investigated.

Twenty patients undergoing living-donor renal transplantation were recruited from a larger cohort of patients participating in a randomized, double-blind trial. All patients were allocated to a single intraoperative dose of either placebo or rituximab (375 mg/m^2) added to the standard immunosuppressive therapy. Blood was collected at baseline, at one day, and at one month after surgery. The healthy kidney donors also gave blood at baseline. Peripheral blood mononuclear cells were stimulated ex vivo in different manners (heat killed Candida albicans yeast, heat killed Staphylococcus aureus, or CD3 CD28 coated beads), to address the role of B cells in ex vivo cytokine responses. The concentration of monocyte- and T cell-derived cytokines (IL-1ß, IL-6, TNF , IFNγ, IL-17 and IL-22) was measured in supernatants.

Of the 20 recruited patients, 13 received treatment with rituximab and 7 received placebo. In all patients, IL-17 was produced by CD4-positive, γ -TCR-negative cells. After stimulation, there was no difference between patients and healthy controls in ex vivo production of IL-17 or other cytokines. In all patients there was a general decrease of monocyte- and T cell-derived cytokines after transplantation, except for IL-17. There was no difference between patients who received rituximab and patients who received placebo.

A single dose of rituximab treatment added to standard immunosuppressive therapy in renal transplant patients did not influence the production of IL-17 or other monocyte- or T cell derived cytokines after ex vivo stimulation.

Introduction

Since the early 2000's, Th17 cells have been recognized as a separate T helper subset characterized by the expression of interleukin (IL)-17. IL-17 is a proinflammatory cytokine that induces chemokine production and promotes recruitment of neutrophils to the site of inflammation. Although their origin and precise function remain incompletely defined, recent studies have shown that Th17 cells are involved not only in the immune response against fungi and extracellular bacteria, but also in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis [192-194]. We recently found that treatment with the B cell depleting antibody rituximab in patients with RA leads to a reduction of Th17 cells in synovial tissue [5]. Moreover, we showed that stimulation of peripheral blood mononuclear cells (PBMCs) in vitro in the presence of rituximab decreases Candida-induced IL-17 production and the number of IL-17 producing cells. These results indicate that B cells contribute to the Candida-induced Th17 response, possibly mediated by their antigen presenting potential or by the production of specific cytokines.

In renal transplantation, T cells play an important role in allograft rejection. During rejection episodes the inflammatory infiltrates contain CD4+ and CD8+ T cells, and antibody therapies directed against T cells, are effective in the prevention and treatment of allograft rejection [195]. Initially, T helper 1 (Th1) cells were believed to be the main T helper subset driving allograft rejection, based on increased urinary protein levels of the IFNγ inducible chemokines MIG (CXCL9) and IP10 (CXCL10), and increased T-bet mRNA levels in renal tissue during allograft rejection [196, 197]. However, recently this idea has been challenged since IFNγ knockout mice experience accelerated graft rejection and increased parenchymal necrosis [198, 199]. Interestingly, IFNγ can inhibit Th17 cells in vitro, suggesting that IL-17 can play an important role in allograft rejection [200, 201]. Indeed, in a mouse cardiac transplantation model, blocking or deficiency of IL-17, in mice mainly produced by γ -T cells, resulted in significantly increased graft survival [202, 203]. Loong et al. reported elevated IL-17 mRNA and protein levels in infiltrating mononuclear cells in renal biopsies and in mononuclear cells from urinary sediments from patients with borderline kidney graft rejection [204]. Furthermore, van Kooten et al. demonstrated the presence of IL-17 mRNA and protein in graft-infiltrating CD4+ and CD8+ T cells in biopsies of acutely rejected human kidney grafts [205].

Based on our previous findings, we hypothesized that B cell depletion might interfere with the induction of Th17 cells and IL-17 production after renal transplantation. In that case, addition of rituximab to the immunosuppressive treatment might improve the outcome of transplantation. In a clinical trial we are evaluating the effectiveness and safety of rituximab for the prevention of acute rejection after renal transplantation. This provided the opportunity, to study cytokine production upon ex vivo stimulation of PBMCs obtained from a subgroup of patients who participated in the abovementioned trial. We expected to find that the in vivo depletion of B cells, would reduce the ex vivo Candida or Staphylococcus aureus induced IL-17 production by PBMCs. Reporting on the main outcome of the clinical trial is beyond the scope of this manuscript and will follow once the trial has been completed. We hypothesize that in vivo B cell depletion will decrease ex vivo IL-17 production in cells from renal transplant patients.

Materials and methods

Subjects

Between April 2009 and May 2010, twenty patients were recruited from a cohort of patients participating in a randomized, double-blind, placebo-controlled trial evaluating the effectiveness and safety of rituximab for the prophylaxis of acute allograft rejection after renal transplantation. In the clinical trial, patients were treated either with a single intraoperative dose of rituximab (375 mg/m^2) or placebo, added to standard immunosuppressive therapy, consisting of tacrolimus, mycophenolate mofetil and prednisone. Patients did not receive induction therapy with anti-T cell agents. A detailed description of this trial can be found on Clinicaltrials.gov, trial number NCT00565331. For logistic purposes, only patients that underwent a planned renal transplantation (e.g. only those with a living donor) were included in the current study on ex vivo cytokine production.

For ex vivo cytokine stimulation, venous blood was collected at baseline i.e. a few hours (maximum 6) before surgery, 24 h later, and one month after the operation by venipuncture into 10 mL EDTA tubes (367525, BD, Plymouth, UK). Moreover, to evaluate B cell depletion, 10 mL of blood was also collected at one week after surgery. When a biopsy-proven rejection or graft loss occurred, only the samples collected before this event were analyzed. The living kidney donors of each recipient acted as healthy controls and in these subjects blood was drawn 1 to 2 h before their surgery. The parent

study was approved by the Central Committee on Research involving Human Subjects (The Hague, The Netherlands) and the Medical Research Ethics Committee Arnhem-Nijmegen (Nijmegen, The Netherlands). All patients gave written informed consent for the parent study, which also implied consent for the current study.

Reagents

The following materials were used for the isolation of PBMCs: Ficoll-Paque (GE Healthcare, Diegem, Belgium); RPMI 1640 Dutch modifications (Sigma-Aldrich) supplemented with 1% gentamicin, 1% l-glutamine and 1% pyruvate (Life Technologies, Nieuwekerk, The Netherlands). The following stimuli were used to induce a strong Th17 response in vitro [206]: Anti-CD3 and anti-CD28 coated (CD3 CD28) beads were prepared from a T cell activation/expansion kit (MACS Milteny Biotec, Bergisch Gladbach, Germany); Candida albicans ATCC MYA-3573 (UC 820) was grown overnight in Sabouraud broth at 37 C [207]. C. albicans was heat-killed (HK) for 1 hour at 100 C; and S. aureus (ATCC) 25923 was heat-killed for 30 minutes at 100 C. The following antibodies were used for FACS analysis: -TCR PAN -PE (Beckman Coulter, Breda, The Netherlands), CD4-FITC (BD Pharmingen, Erembodegem, Belgium), IL-17-Alexa647 (BD Pharmingen), CD19-APC-Alexa Fluor 750 (Beckman-Coulter) and CD45-Krome Orange (Beckman-Coulter).

Ex vivo cytokine production

PBMCs were isolated by density gradient centrifugation of PBS-diluted blood (1:1) over Ficoll-Paque, washed twice with saline, and suspended in culture medium. The cells were counted in a Coulter Counter (Beckman Coulter, Woerden, The Netherlands). 5 10^5 PBMCs in a volume of 100 µL per well were incubated at 37 C in round-bottom 96-well plates (Greiner, Nurnberg, Germany). PBMCs were primed, for one, two or seven days, with RPMI, C. albicans, S. aureus or CD3 CD28 beads. These stimuli are known to induce high IL-17 production in PBMC. Anti-CD3/anti-CD28 can induce IL-17 production in T cells without the need for any other co-stimulatory signals and/or antigen presentation. In contrast, both HK C. albicans and S. aureus require to be processed by antigen-presenting cells first, before they can induce IL-17 production in T cells. In blocking experiments, PBMCs were preincubated for 1 h with 10 µg/mL rituximab. When cells were cultured for seven days, this was done in the presence of 10% human serum. After one, two or seven days, supernatants were collected and stored at 20 C until assayed.

Flow cytometry IL-17+-cells

PBMCs were stimulated for 7 days as described above. After 7 days the supernatant was removed and replaced with RPMI with Golgiplug (555029, BD Biosciences, Breda, The Netherlands), PMA (50 ng/mL) (P8139, Sigma-Aldrich), and ionomycin (1 µg/mL) (I0634, Sigma Aldrich) for 4 h. Subsequently, the cells were labeled with anti-CD4-FITC and anti-γ -TCR-PE according to the instructions of the manufacturer. Fix & Perm (GAS001S-100 and GAS002S-100, Invitrogen) were used to stain intracellularly with anti-IL17-Alexa647. The cells were resuspended in 200 µL 1% BSA and fluorescence was measured on a Cytomics FC500 FACS machine (Beckmann Coulter, Woerden, The Netherlands).

Flow cytometry B cells

PBMCs were isolated by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Roskilde, Denmark) and cryopreserved in liquid nitrogen until analysis. Cells were labeled with CD19 (J3-119)-APC-Alexa Fluor 750 and CD45-Krome Orange. The cell phenotype was analyzed by ten-color flow cytometry (Navios) and data were analyzed using Kaluza software (all from Beckman-Coulter).

Cytokine assays

The concentrations of IL-1ß, IL-17, IL-22 and TNF (R&D Systems, Abingdon, UK) and IL-6 and IFNγ (Sanquin, Amsterdam, The Netherlands) were measured in cell culture supernatants using ELISA, according to the instructions of the manufacturer. Based on previous experience, IL-1ß, IL-6 and TNF were measured after 24 h of stimulation, IFNγ after 48 h and IL-17 and IL-22 after seven days of stimulation [206].

Statistical analysis

Experiments were performed in duplicate, and supernatants of the duplicates were pooled. The differences between groups were analyzed using the Wilcoxon rank-sum test (**Figure 2 and Figure 3**). Trends in cytokine production over time were analyzed using repeated measures analysis (**Figure 3**). Violation of sphericity (Mauchly's $p < 0.05$) was corrected using the Greenhouse–Geisser correction. All p values were adjusted for multiple testing, using the Bonferroni correction. The level of significance was set on $p < 0.002$ (**Figure 2**), $p < 0.002$ (main ANOVA **Figure 3**), $p < 0.004$ (contrasts **Figure 3**), and $p < 0.0007$ (**Figure 3**). Data are presented as mean ± SD (standard deviation).

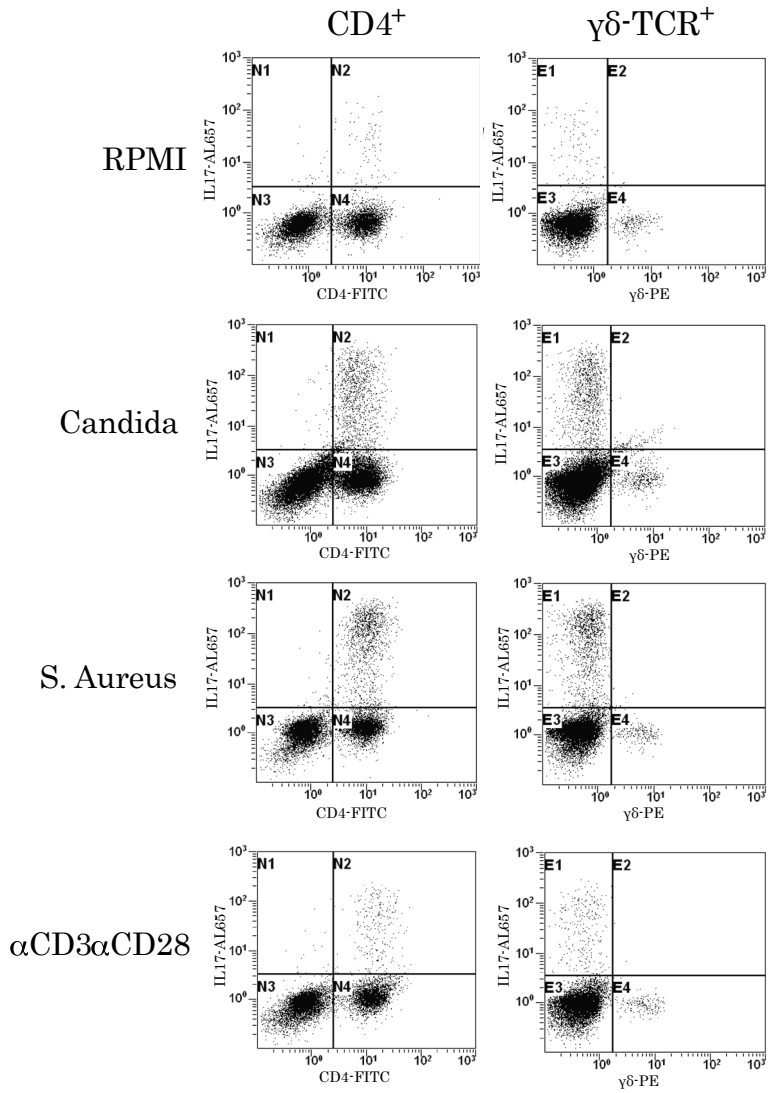
Results

Between April 2009 and May 2010, we included 20 patients (fourteen males and six females, age (mean ± SD): 47 ± 12 years). Ten patients were treated with hemodialysis before transplantation, two with peritoneal dialysis, and eight underwent a pre-emptive transplantation. Thirteen patients were treated with rituximab and seven with placebo. One patient lost the graft due to venous thrombosis the day after surgery, and was excluded from further analysis. One week after surgery the percentage of CD19+ B cells within the CD45+ lymphocyte fraction was (mean ± SD) 0.24 ± 0.22 in patients who had received rituximab, compared to 23.0 ± 21.9 in patients who had received placebo during surgery ($p < 0.01$). Three patients had biopsy-proven rejection, all within the first month after transplantation, and were excluded from the analysis at one month after surgery. The 20 healthy kidney donors (seven males and thirteen females) had an age (mean ± SD) of 55 ± 10 years.

In order to determine the cell type responsible for IL-17 production in our in vitro system, PBMCs were stimulated for 7 days, in the presence of 10% human serum, with HK C. albicans, S. Aureus, or anti-CD3/anti-CD28. Intracellular IL-17 was measured using FACS. IL-17 expression could especially be detected after C. albicans and S. aureus stimulation. All IL-17 positive cells were CD4 positive and γ -TCR negative (**Figure 1**).

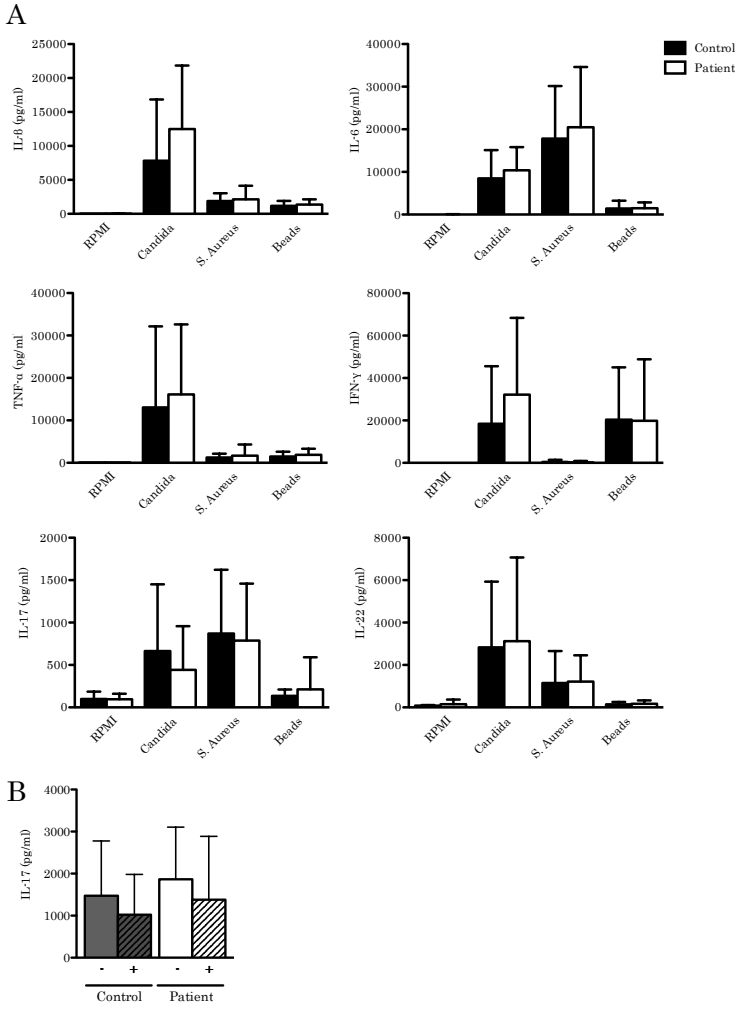
First we wanted to assess whether renal transplant recipients are comparable to healthy controls to rule out any effect on cytokine production caused by the underlying kidney disease. There were no differences in the ex vivo production by the PBMCs of IL-1ß, IL-6, TNF , IFNγ, IL-17 and IL-22 upon stimulation with HK C. albicans yeast, HK S. aureus, or anti-CD3/anti-CD28 coated beads between the 20 patients and the 20 healthy kidney donors (**Figure 2A**). Furthermore, in vitro pre-incubation with rituximab decreased Candida-induced IL-17 production in healthy controls to a similar extent in renal transplant recipients (**Figure 2B**).

Figure 1. Source of IL-17 production in vitro.



Human PBMC were stimulated for 7 days with C. albicans (1 10^6/mL), Staphylococcus aureus (1 10^7/mL), or anti-CD3/anti-CD28 beads (1.25 10^6/mL), in the presence of 10% human serum. Expression of CD4, γ -TCR and intracellular IL-17 were determined with FACS analysis. Figure is representative for four healthy volunteers from two different experiments.

Figure 2. Cytokine production in patients with end-stage renal failure versus healthy controls.



(A) Human PBMCs isolated before surgery were stimulated with RPMI, C. albicans (1 10^6/mL), Staphylococcus aureus (1 10^7/mL), or anti-CD3/anti-CD28 beads (1.25 10^6/mL). Cytokine concentrations were measured in cell culture supernatants using ELISA. IL-1β, IL-6 and TNF were measured after 24 hours of stimulation, IFNγ after 48 hours, and IL-17 and IL-22 after seven days of stimulation. When cells were stimulated for seven days, this was done in the presence of 10% human serum. Experiments were performed with cells from 20 healthy volunteers and 20 patients with end-stage renal failure. Data are presented as mean ± SD.

(B) Human PBMCs from five healthy volunteers and five patients with end-stage renal failure, isolated before surgery, were stimulated for seven days in the presence of 10% human serum with C. albicans (1 10^6/mL), in the absence (-) or presence (+) of rituximab (10 μg/mL). IL-17 was measured in cell culture supernatants using ELISA. Data are presented as mean ± SD.

In all patients, there was a general decrease of production of IL-1β, TNF , and IFNγ at the day after surgery and one month later (**Figure 3**). A recovery was seen in the production of IFNγ, but only in the case of stimulation with the anti-CD3/anti-CD28 coated beads. The production of IL-6 and IL-22 was also decreased at one day after surgery, but was mostly recovered at one month after transplantation. Notably, the general decrease in cytokine production at day one after surgery was not observed for IL-17. Candida-induced IL-17 production tended to increase the day after transplantation, and even more at one month after transplantation, although this rise was not statistically significant (**Figure 3**).

Despite a nearly complete B cell depletion in the rituximab group, there was no difference in cytokine production (with any stimulus at any time point) between patients treated with rituximab and patients treated with placebo (**Figure 3**). Cytokine production, including IL-17 production, was not associated with the occurrence of rejection (n = 3), the occurrence of one or more infections (n = 15), or baseline patient characteristics, although the numbers were too small for statistical analysis (data not shown).

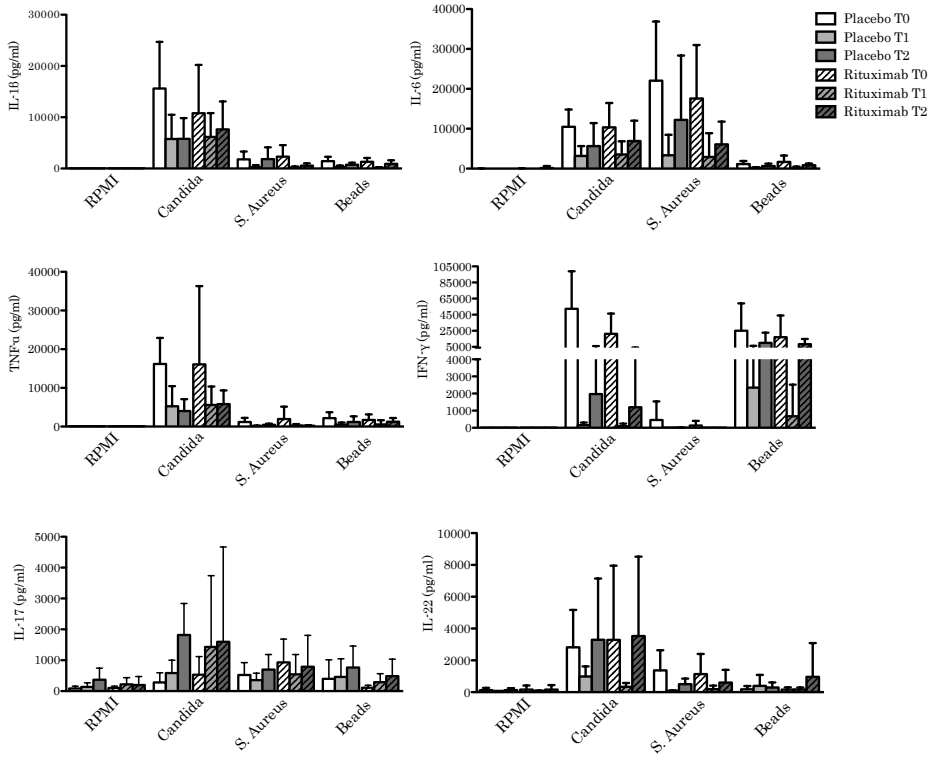
Discussion

In this study, administration of a single dose of rituximab resulted in nearly complete B cell depletion, but did not have an effect on ex vivo cytokine production, including IL-17. We therefore could not confirm our hypothesis that B cells contribute to the in vivo Th17 response.

We previously showed that in vitro addition of rituximab to PBMCs obtained from healthy individuals reduces the IL-17 inducing potential of C. albicans [5]. After reconfirming these findings, we evaluated whether in vivo B cell depletion by rituximab, which is clinically more relevant, also results in a reduced capacity of PBMC to produce IL-17 and other cytokines upon ex vivo stimulation. Despite a profound in vivo B cell depletion, we could not reproduce the effects observed after a similar degree of B cell depletion upon adding rituximab in vitro. The detection of an effect of rituximab treatment on ex vivo cytokine production might have been hampered by the general decrease in cytokine responses that we observed after transplantation. This decrease may be related to the use of the immunosuppressive drugs tacrolimus, mycophenolate mofetil, and steroids. The decrease at 24 hour after transplantation could be related to

the occurrence of a short-lived cytokine release syndrome after the administration of rituximab or the use of clemastine and steroids before the infusion of rituximab/placebo [177]. Both mechanisms might prohibit the production of cytokines when PBMCs are (re)stimulated ex vivo [208].

Figure 3. Effect of rituximab on *ex vivo* cytokine production in renal transplant patients.



Human PBMCs isolated before (T0), one day after (T1), or one month (T2) after renal transplantation, were stimulated with RPMI, *C. albicans* (1 10^6/mL), *Staphylococcus aureus* (1 10^7/mL), or anti-CD3/anti-CD28 beads (1.25 10^6/mL). Cytokine concentrations were measured in cell culture supernatants using ELISA. IL-1β, IL-6 and TNF were measured after 24 hours of stimulation, IFNγ after 48 hours, and IL-17 and IL-22 after seven days of stimulation. When cells were stimulated for 7 days, this was done in the presence of 10% human serum. Experiments were performed with cells from 13 rituximab-treated patients and 7 placebo-treated patients (T0), or 13 rituximab-treated patients and 6 placebo-treated patients (T1), or 11 rituximab-treated patients and 5 placebo-treated patients (T2). Data are presented as mean ± SD.

Interestingly, we found no decrease in IL-17 production, both at one day and at one month after transplantation. Previously it was shown that treatment of myasthenia gravis patients with tacrolimus resulted in a reduced production of IFNγ and IL-17 by PBMCs [209]. Therefore, the finding that IL-17 production was not suppressed in this study (in which all 20 patients were treated with tacrolimus next to mycophenolate mofetil and steroids) is remarkable. However, it is difficult to dissect the separate effects of different drugs on ex vivo cytokine production. Furthermore, regulation of IL-17 production is quite different from other proinflammatory cytokines. For example, IL-17 production is increased by PGE2, in contrast to the production of TNF and IFNγ, which are suppressed by PGE2 [210-214]. Regardless of the exact mechanism of a lack of IL-17 suppression, our observation pinpoints IL-17 as a potential novel target for prophylaxis against graft rejection.

We were not able to show a correlation between ex vivo IL-17 production and the occurrence of graft rejection. However, rejection occurred in only three patients, which precludes statistical analysis. Notably, we also found an increase in IL-17 production in PBMCs from patients without graft rejection.

In conclusion, treatment with a single peri-operative dose of rituximab added to the standard immunosuppressive therapy does not influence ex vivo cytokine production, including IL-17. We therefore could not confirm our hypothesis that B cell depletion might interfere with the induction of Th17 cells.

Chapter 10

Summary and general discussion

For patients with end-stage renal disease a renal transplantation is the best option for improvement of quality of life and life expectancy and is therefore generally preferred over treatment with dialysis. Over the last decades, great improvement has been achieved, but important issues remain to be solved.

The first issue investigated in this thesis is the high incidence of delayed graft function in patients transplanted with a kidney donated after circulatory death. In the study presented in **chapter 4** it is shown that a single intraoperative dose of ATG-Fresenius (ATG-F) added to standard triple immunosuppressive regimen, with an unadjusted tacrolimus dose, was not effective to reduce the incidence or duration of delayed graft function and was associated with a higher incidence of serious adverse events. This is in contrast to other studies with ATG-F in which a reduced incidence of delayed graft function was seen [86, 126-131]. However, in these studies, the addition of ATG-F was accompanied by a reduction in the tacrolimus dose. In such a trial design it is unclear whether the beneficial effect on delayed graft function should be attributed to the addition of ATG-F or to the reduction of the tacrolimus dose. For this reason we used an unadjusted tacrolimus dose in our trial.

The lack of effect of ATG-F on delayed graft function questions the effectiveness of ATG-F itself. Although we noticed lymphocytopenia and mild thrombocytopenia in ATG-F-treated patients, we did not find an effect on the incidence of allograft rejection. In other trials with ATG-F and also with ATG-Thymoglobulin, this was universally the case. This lack of effect on both delayed graft function and allograft rejection argues for insufficient effectiveness of ATG-F.

We noticed a higher incidence of serious adverse events and a trend towards more infections in the ATG-F-treated patients. This study was not performed in a blinded fashion and therefore a bias towards reporting side effects in ATG-F-treated patients could have occurred. A higher incidence of infections has not been reported in other studies with ATG-F and could be the consequence of using an unadjusted dose of tacrolimus in combination with ATG-F. In conclusion, the use of ATG-F without adjustment of the tacrolimus dose cannot be recommended to reduce the incidence of delayed graft function in patients transplanted with a DCD-donor kidney.

Currently, much research is directed to the prevention of delayed graft function after renal transplantation: a search on clinicaltrials.gov on 'delayed graft function' and 'renal' resulted in more than 40 running clinical trials. Most trials investigate currently available drugs, like eculizumab, a monoclonal antibody targeting the complement

system. At Mount Sinai Hospital (New York), a trial has recently been started which evaluates the effect of eculizumab on the incidence of delayed graft function within the first seven days after renal transplantation (clinicaltrials.gov number NCT01919346). Patients transplanted with a kidney at high risk of severe ischemia-reperfusion injury (ischemia time more than 18 hours or kidneys from donors aged 60 years or older, or aged 50-59 years with comorbidity) will be randomized to two doses of eculizumab or placebo. Unfortunately, donors after circulatory death, the type of donor used in our trial, are excluded.

Another approach to reduce the incidence of delayed graft function could be the interference with the toll-like receptors. They form a group of receptors that recognize pathogen- or damage-associated molecular patterns. One of these receptors is toll-like receptor 2 (TLR2), which is expressed on innate immune cells and renal epithelial cells and can be activated by ligands that are released following ischemic injury. Experimental data indicate that activation of TLR2 is involved in the inflammatory cascade leading to ischemia-reperfusion injury. Specific inhibition of TLR2 by a monoclonal antibody named OPN-305 could therefore reduce the incidence of delayed graft function [210]. A placebo-controlled, double blind trial with OPN-305, has been initiated in various countries. In this study, next to recipients of a DBD kidney with a long cold ischemia time or from extended criteria donors, patients receiving a kidney from a donor after circulatory death are included (NCT01794663).

Finally, a different and possibly more attractive approach to alleviate the problem of ischemia-reperfusion injury is to reduce ischemia time by more rapid allocation and transportation of organs. Nevertheless, there will always remain a time interval between organ procurement and implantation in the recipient. The usual way to protect organs during this period is to store them at melting ice after perfusion with a preservation fluid. An alternative is continuous, pulsatile perfusion with a cold (1-8 °C) preservation fluid. In a recent trial one kidney of each donor was randomly assigned to hypothermic machine perfusion and the contralateral kidney to cold storage. Machine perfusion was associated with a substantial reduction in the incidence of delayed graft function [215]. An even more physiological approach would be normothermic (and oxygenated) machine perfusion, and the first small series in renal transplantation demonstrates that this technique is both feasible and safe [216].

Chapter 5 reports that alemtuzumab appeared to be equally effective as ATG-Thymoglobulin in the treatment of steroid-resistant renal allograft rejection, with a more

favorable side effect profile. However, these results have to be interpreted with caution, since these data were not derived from a prospective trial. Baseline characteristics of both groups were not completely comparable, mainly because the alemtuzumab groups contained more patients with a retransplantation. These patients had been treated with ATG during a previous transplantation, and the avoidance of repeated exposure to ATG was the main reason for treatment with alemtuzumab. Nonetheless, this imbalance would be expected to favor the ATG group, since patients undergoing a retransplantation may be more sensitized and therefore more difficult to treat. Another point of concern is the limited follow-up of three months. The T cell depleting effect of ATG lasts approximately 12 months. This can even be longer after alemtuzumab treatment. Late side effects, especially infections or malignancies could therefore occur later than three months after administration of either drug. Consequently, this study does not provide sufficient evidence to recommend alemtuzumab as the first-line therapy in steroid-resistant rejection. A formal, randomized controlled trial, with long-term follow-up is warranted. Of special interest is a recent report in which the use of alemtuzumab appears to be associated with an increased risk of the development of donor-specific HLA-antibodies [217]. This could negatively influence long-term graft survival. Despite these concerns, alemtuzumab can be considered the treatment of choice for patients with a steroid-resistant rejection in whom ATG is an unattractive option. This can be based on prior ATG administration or on cardiopulmonary problems or other comorbidity which might jeopardize the patient when a first-dose reaction after administration of ATG would occur.

As mentioned above, a drawback of both ATG and alemtuzumab is the long lasting non-specific depletion of T cells. Ideally, new antibodies should be developed which target only alloreactive T cells without long-lasting depletion of non-alloreactive cells and without causing cytokine release. Currently, no trials are planned or ongoing with anti-T cell antibodies in established allograft rejection, although anti-T cell antibodies (mainly directed against CD3) are currently under investigation in auto-immune diseases like type 1 diabetes mellitus.

Although equally effective in treating steroid-resistant allograft rejection, the mechanism of action of ATG and alemtuzumab is not identical. The antibodies present in ATG are mainly directed against T cell-specific molecules like CD2, CD3, and CD8, although a variety of other of antibodies directed to antigens expressed on NK cells and endothelial cells are also present [163, 218]. Some studies show no significant amount of antibodies against B cell markers like CD19 or CD20 in ATG. However, ATG has anti-B

cell properties, either directly by depletion through different receptors, like CD5 or HLA-DR, or indirectly by eliminating CD4+ T cell help [16, 218]. In contrast, alemtuzumab targets specifically the CD52 molecule, which is expressed on T, NK cells, monocytes, macrophages, dendritic cells, and on B cells. This specific targeting could contribute to the clinical effect of alemtuzumab in treating steroid-resistant rejection. The increasing knowledge on the role of B cells and antibodies in graft rejection has led to growing attention to anti-B cell therapy. Nonetheless the number of clinical trials with anti-B cell therapy in renal transplantation is limited.

In **chapter 6** we present the data of a randomized clinical trial in which the effect of anti-B cell therapy on the incidence of acute allograft rejection was investigated. A single dose of rituximab or placebo was added to standard maintenance immunosuppression of tacrolimus, mycophenolate mofetil and steroids in 280 renal transplant patients. Although treatment with rituximab was safe, it was ineffective to reduce the incidence of acute rejection in a broad population of renal transplant patients. During the design of the trial, we hypothesized that the effects of rituximab could be different between subgroups of patients. Therefore we stratified our included patients for the presence of anti-HLA antibodies (as reflected by the panel reactive antibody (PRA) value) and rank order of transplantation. When these strata were combined to form an immunologically low-risk (PRA 6% and first transplantation) and an immunologically high-risk (PRA>6% or retransplantation) population, a clear trend towards a lower incidence of acute allograft rejection with rituximab therapy as compared to placebo was observed in the immunologically high-risk subgroup. Interestingly, the incidence of antibody mediated rejection tended to be lower after rituximab, especially in immunologically high-risk patients. However, the study was not sufficiently powered for these analyses, and therefore caution is required. Nonetheless, these results suggest a protective effect of rituximab against acute rejection in patients who are at higher immunological risk. This can possibly be explained by depleting the memory B cells, which were present in higher frequencies in this group. With the current median duration of follow-up of 4 years, the beneficial effect on allograft rejection incidence has not resulted in improved graft function or graft survival. Furthermore, it remains to be answered whether treatment with rituximab can reduce the development of donor-specific anti-HLA antibodies (DSA). While the routine measurement of DSA posttransplant was not part of standard clinical care at the time of the initiation of the current study and was not included as a study endpoint, the results of measurement of these antibodies in stored follow-up sera will become available in the near future.

An aspect that deserves attention is the dose and intensity of rituximab therapy. The lack of effect in the broad population of renal transplant patients could justify an intensified (increased or repeated dose) treatment regimen. It has been demonstrated that a single dose of rituximab, depletes B cells in peripheral blood, but not in secondary lymphoid organs and it is known that repeated doses of rituximab are required for full depletion of B cells from lymph nodes [146, 219]. However, we noticed that after a single dose of rituximab B cell depletion in the peripheral blood lasted more than 12 months. An intensified regimen could lead to even longer B cell depletion, potentially increasing the risk of unwanted effects like infections or malignancies. On the other hand, the minimal dose at which rituximab leads to B cell depletion appears to be much lower than the usual dose of 375 mg/m^2. A Japanese study has shown that doses as low as 15-35 mg/m^2 were still able to cause prolonged B cell depletion in the peripheral blood and spleen [220]. Lower doses could not only be economically attractive, but could also lead to a shorter period of B cell depletion and the effect of shorter B cell depletion is not known. Given these and the above-mentioned issues, it would be premature to recommend the incorporation of rituximab into clinical practice in immunologically high-risk patients.

The incidence of infections and malignancies was not increased in rituximab-treated patients despite a prolonged B cell depletion in the peripheral blood and a transient neutropenia in about 25% of patients. The absolute incidence of infections was high (75-80% of patients had one or more infections within the first 24 months posttransplant) in both groups, which could obscure an additional effect of rituximab. However, we did not find a difference in the type or severity of infections and length of hospitalization. The absence of a clear increase in the incidence of infections after treatment with rituximab is remarkable since B cells are required for an optimal defense against microbial pathogens. For example, inborn errors of B cell development result in agammaglobulinemia, leading to recurrent (upper) respiratory tract infections [221]. An explanation for our findings could be that a single dose of rituximab only partially depletes B cells. It has been demonstrated that a single dose of rituximab, depletes B cells in peripheral blood, but not in secondary lymphoid organs [146]. More-over, in other trials in renal transplant patients, treatment with rituximab was not associated with a decrease in immunoglobulin levels [106]. Altogether, despite these concerns, a single dose of rituximab appears to be safe.

During the last years it has become clear that specific B cell subsets, characterized by the ability to produce IL-10, can have immunoregulatory effects resulting in a potential protective effect against graft rejection [222, 223]. The demonstration of elevated

numbers of naive and transitional B cells in the blood and an upregulation of CD20 mRNA in renal cells extracted from urine of drug-free transplant patients supports the regulatory function of certain B cell subsets [224, 225]. Non-selective B cell depletion could therefore also lead to depletion of these immunoregulatory B cells, with potentially deleterious effects.

Next to the discovery of B cell subsets, our understanding of the factors that determine B cell activation, differentiation, and proliferation has increased. Specific B cell growth factors such as BLyS (B lymphocyte stimulator) and APRIL (a proliferation inducing ligand) play an important role in B cell development and survival [226-228]. In a murine cardiac transplant model, BLyS-deficient mice show prolonged cardiac allograft survival [229]. In vivo BLyS neutralization effectively induced tolerance and promoted long-term islet allograft survival in mice [230]. In humans, increased BLyS levels appeared to be a risk factor for renal allograft dysfunction and development of donor-specific antibodies [231]. Moreover BLyS-receptor positive T and B cells have been found in allografts with chronic rejection [229]. Therefore, new therapies that (additionally) target BLyS could be beneficial to prevent allograft rejection. In clinical trials, belimumab, a fully human anti-BLyS antibody, has been investigated in severe lupus arthritis. Belimumab was given at monthly doses for 72 weeks. In a dose dependent manner, it led to a significant reduction in disease activity, relapse rates and reduced requirement of steroids compared to placebo. The incidence of serious infections was not increased [232]. The benefits of this kind of anti-B cell intervention to prevent allograft rejection in renal transplantation are currently investigated in a clinical trial in Cambridge (NCT01536379). Given the results of our rituximab study, trials testing newer anti-B cell strategies should especially focus on immunologically high-risk patients. The endpoints of these studies should include long-term safety and graft survival and the presence of (donor-specific) anti-HLA antibodies.

Rituximab can effectively deplete B cells from the peripheral blood, as we have shown in chapter 6. However in lymph nodes a B cell population remains after administration of a single dose of rituximab, although this population has different functional capacities [146]. The question whether rituximab can reduce B cell infiltration of the allograft during rejection, was investigated in **chapter 7.** To this end, the extent of B cell infiltration was scored in biopsies showing acute rejection according to the Banff classification. In patients treated with the standard immunosuppressive regimen consisting of tacrolimus, mycophenolate mofetil and steroids, we found varying degrees of B cell infiltration. However, when rituximab was added as induction therapy, the

rejection biopsies contained virtually no B cells. No difference was seen in the severity of tubulitis, arteritis or the extent of the cellular infiltrate in rituximab-treated patients, as compared to placebo-treated patients. In previous studies, the presence of intragraft B cells during acute allograft rejection was associated with a higher chance of steroid-resistance of the rejection and of subsequent graft loss. In our cohort, the lack of B cell infiltration in rituximab-treated patients was not associated with a better response to steroids or improved graft outcome. This questions the pathophysiological role of intragraft B cells in the acute phase of allograft rejection. Our data suggest that the presence of intragraft B cells during acute allograft rejection could be considered an epiphenomenon rather than a contributing pathological factor.

Activation of B cells can lead to the release of a wide range of cytokines [179]. Treatment with rituximab in lymphoma patients who have high B cell counts, is well known for a clinical syndrome of fever, chills and hypotension, caused by a massive release of cytokines [137]. In **chapter 8** it is evaluated whether a comparable cytokine release occurs after rituximab infusion in renal transplant patients. We indeed found that cytokines were released in the serum, especially IL-10 and MIP-1ß, but none of the patients experienced clinical signs of a cytokine release syndrome. Compared with placebo-treated patients, rituximab-treated patients had increased levels of IL-10 and MIP-1ß at 2 and 4 hours after the start of the infusion. Within 24 hours, the concentrations of these cytokines had returned to baseline levels. In additional in vitro experiments where rituximab was added to various populations of peripheral blood mononuclear cells (PBMCs) we found that MIP-1ß was only secreted when rituximab was added to a co-culture of B cells and NK cells. Intracellular staining of MIP-1ß showed that NK cells, and not B cells produce MIP-1ß. Incubation of PBMCs with rituximab-F(ab')$_2$ did not lead to an increased MIP-1ß secretion, indicating that the cytokine release was Fc-receptor dependent.

The transient increase in IL-10 and MIP-1ß levels could be of clinical relevance, since this might modulate the alloimmune response thereby influencing the transplantation outcome. IL-10 is generally thought to be an anti-inflammatory cytokine [189]. The role of MIP-1ß appears to be more ambivalent. In mouse models it was shown to be the most potent chemoattractant for regulatory T cells, but in other models MIP-1ß secreted by NK or B cells recruited monocytes, T cells, and dendritic cells to the site of injury or inflammation via the chemokine receptor CCR5 [190, 191]. Therefore, the release of IL-

10 and of MIP-1ß can have opposing effects on the immune response and the clinical consequences of these findings are yet unclear.

In the final study of this thesis, presented in **chapter 9**, the attention is focused on the interaction of B cells with T cells. Nowadays, it is obvious that there is no absolute lineage commitment of T cells, but that T cell subset show considerable plasticity driven by numerous signals, including different interleukins [233]. Th17 cells are a relatively recently described subset of T cells, characterized by the production of certain cytokines, especially IL-17 and IL-21. Th17 cells play a role in the protection against extracellular pathogens, are involved in various auto-immune diseases, and recent data suggest that they might also play a role in allograft rejection. Based on previous studies we hypothesized that B cells are involved in the induction of a Th17 response. To test this hypothesis, we obtained PBMCs in a subset of patients participating in our rituximab trial. We stimulated PBMCs ex vivo and measured different monocyte and T cell derived cytokines. Although in all patients the production of most cytokines was decreased after transplantation as compared to before transplantation, no additional effect of rituximab on ex vivo cytokine production was observed, despite a profound B cell depletion. These findings indicate that B cells are not required for the induction of a Th17 response, which does not fit with a previously observed decrease in the Th17 response after in vitro addition of rituximab to normal PBMCs [5]. However, we administered rituximab in an in vivo setting which did fundamentally differ from the controlled in vitro environment. One of the differences is the use of the immunosuppressive drugs tacrolimus, mycophenolate mofetil, and steroids, next to the additional use of antihistamines [177, 208]. The use of these drugs could explain why a general decrease in cytokine production was found after the transplantation. Additionally in rituximab-treated patients, the occurrence of short-lived cytokine release after the administration of rituximab (with increased levels of IL-10 and MIP-1ß as found in chapter 8) could have exhausted pre-formed stores of cytokines and could thereby prohibited the release of cytokines when PBMCs are (re)stimulated ex vivo. An interesting finding in this study was that ex vivo IL-17 production was not decreased, both at one day and at one month after transplantation, despite the use of the abovementioned immunosuppressive drugs. If a pathophysiological role of IL-17 and Th17 cells in renal allograft rejection becomes more established in the future, these data suggest that different immunosuppressive strategies are necessary to suppress the IL-17 response and that rituximab will probably not be beneficial to achieve this goal.

To conclude, the aim of this thesis was to investigate the safety and efficacy of novel approaches in renal transplantation. We found that ATG-F was ineffective to reduce the incidence of delayed graft function, and was associated with an increased incidence of severe adverse events. Therefore different strategies to reduce the incidence of delayed graft function are necessary. In patients with a steroid-resistant rejection, ATG-Thymoglobulin will remain the first-line treatment, although alemtuzumab was effective and safe and can therefore be considered a reasonable alternative when there are anti-ATG antibodies or when treatment with ATG is perceived too harmful. Our trial with rituximab to deplete B cells has shown that only a subset of immunologically high-risk patients might benefit from induction therapy with a single dose of rituximab. We did not observe an effect on the incidence of acute allograft rejection in immunologically low-risk patients. Despite an increased incidence of neutropenia, treatment with rituximab was safe and did not lead to an increased risk of infection or malignancies. Rituximab was able to effectively deplete peripheral blood B cells and intragraft B cells, although a beneficial effect of the latter on the allograft affected by acute rejection was not observed. A small but noticeable effect of rituximab on the serum levels of IL-10 and MIP-1β could be found in vivo, while the clinical relevance of this short-lived cytokine release is currently uncertain. Ex vivo, MIP-1ß was secreted by NK cells when rituximab was added to a co-culture of B cells and NK cells, and appeared to be dependent on the interaction of rituximab with Fc-receptors. Finally, in vitro experiments have shown that anti-B cell therapy does not impair the induction of a Th17 response.

The studies presented in this thesis have contributed to the further understanding of the effects of anti-T cell therapy with ATG-F in delayed graft function and the benefits of alemtuzumab in the treatment of steroid-resistant rejection. Furthermore the clinical trial with rituximab and the additional experiments, have provided a better understanding of the role of B cells in the allo-immune response and have demonstrated that anti-B cell therapy could become a novel approach in selected conditions in renal transplantation.

Chapter 11

Samenvatting en algemene discussie

Voor patiënten met eindstadium nierfalen biedt een niertransplantatie de beste verbetering van kwaliteit van leven en levensverwachting en is daarom te verkiezen boven dialyse. Gedurende de afgelopen decennia is er grote vooruitgang geboekt, maar een aantal belangrijke problemen is onopgelost gebleven. In dit proefschrift worden de veiligheid en effectiviteit van nieuwe behandelmethoden van een drietal van deze problemen besproken, te weten de hoge incidentie van het vertraagd op gang komen van de transplantaatnierfunctie, de behandeling van steroïd-resistente acute rejectie en tot slot de preventie van acute rejectie.

Het eerste probleem dat in dit proefschrift is onderzocht is de hoge incidentie van het vertraagd op gang komen van de transplantaatnierfunctie (delayed graft function) bij patiënten die een transplantatie hebben ondergaan met een nier die bij een overleden donor is uitgenomen na een periode van circulatiestilstand (DCD-donor). Uit de studie die in **hoofdstuk 4** wordt beschreven, blijkt dat een eenmalige intra-operatieve gift van antistoffen tegen T cellen (ATG-Fresenius; ATG-F), toegevoegd aan standaard drievoudige immuunsuppressie niet effectief was in het verminderen van de incidentie of de duur van delayed graft function, maar wel geassocieerd was met een hogere incidentie van serieuze bijwerkingen. Deze bevindingen verschillen met andere studies waarin met ATG-F wél een reductie van de incidentie van delayed graft function werd bereikt [86, 126-131]. Echter, in deze studies werd de behandeling met ATG-F gevolgd door behandeling met tacrolimus in een gereduceerde dosis terwijl deze dosisreductie niet plaatsvond in onze studie. Door die studieopzet is het onduidelijk of het positieve effect op de incidentie van delayed graft function aan moet worden toegeschreven aan de ATG-F of aan de reductie van de tacrolimusdosering. Daarom is in onze studie niet voor een gereduceerde tacrolimusdosering gekozen.

Het ontbreken van een effect van ATG-F op de incidentie van delayed graft function trekt de effectiviteit van het middel ATG-F in twijfel. Alhoewel er lymfocytopenie en een milde trombocytopenie optraden, had dit geen effect op de incidentie van acute rejectie. In andere studies met ATG-F trad dit vrijwel altijd wel op. Het ontbreken van een effect van ATG-F op de incidentie van zowel delayed graft function als rejectie wijst op onvoldoende effectiviteit van ATG-F.

Opvallend was de hogere incidentie van serieuze bijwerkingen en de trend naar een hogere incidentie van infecties bij met ATG-F behandelde patiënten. Deze studie was niet geblindeerd, waardoor bijwerkingen bij met ATG-F behandelde patiënten mogelijk eerder en vaker werden gemeld. De hogere incidentie van infecties werd in andere

studies met ATG-F niet gemeld en kan het gevolg zijn van de combinatie van ATG-F met een niet-gereduceerde tacrolimusdosering. Concluderend kan het gebruik van ATG-F met een niet-gereduceerde tacrolimusdosering niet worden aanbevolen om de incidentie van delayed graft function te verlagen bij patiënten die een transplantatie ondergaan met een nier van een DCD-donor.

Momenteel wordt er veel onderzoek verricht naar de preventie van delayed graft function na niertransplantatie; een zoektocht op clinicaltrials.gov leverde meer dan 40 lopende studies op. De meeste studies maken gebruik van reeds bestaande medicamenten, zoals eculizumab, een monoklonaal antilichaam dat de werking van het complement systeem remt. In het Mount Sinai Hospital in New York is recent een studie gestart die het effect van eculizumab op de incidentie van delayed graft function in de eerste week na transplantatie onderzoekt (clinicaltrials.gov nummer NCT01919346). Patiënten die een niertransplantatie ondergaan waarbij het risico op ischemie-reperfusie schade hoog is (donornieren met een koude ischemie tijd van meer dan 18 uur of nieren van donoren 60 jaar en ouder of 50-59 jaar met comorbiditeit), worden gerandomiseerd tussen behandeling met twee doses eculizumab of placebo. Helaas worden patiënten die een nier van een DCD-donor ontvangen, de onderzoekspopulatie van onze studie, niet in deze studie geïncludeerd.

Tot slot zou snellere allocatie en transport van organen een andere, potentieel aantrekkelijkere optie zijn om de incidentie van delayed graft function te verlagen. Er zal echter altijd een tijdsinterval blijven bestaan tussen uitname bij de donor en implantatie van het donororgaan bij de ontvanger. De gangbare preservatiemethode is het bewaren van de nieren op smeltend ijs na perfusie met een koude vloeistof (1-8 °C). Een alternatieve preservatiemethode is continue, pulsatieve perfusie met koude perfusievloeistof met behulp van een machine. In een recente studie is één nier van een donor gerandomiseerd voor machinale perfusie, terwijl de contralaterale nier werd bewaard op smeltend ijs. In deze studie was machinale perfusie geassocieerd met een substantiële reductie van de incidentie van delayed graft function [215]. Een volgende, meer fysiologische, stap is normotherme (en eventueel geoxygeneerde) machinale perfusie. De eerste resultaten bij niertransplantatie laten zien dat deze techniek haalbaar en veilig is [216].

In **hoofdstuk 5** wordt een tweede probleem van orgaantransplantatie beschreven; behandeling van steroïd-resistente. Uit het onderzoek beschreven in dit hoofdstuk blijkt dat de monoklonale antistof alemtuzumab even effectief lijkt als ATG-Thymoglobuline

als behandeling van steroïd-resistente acute rejectie, terwijl het een gunstiger bijwerkingsprofiel heeft. De resultaten zijn echter niet afkomstig van een prospectieve studie en moeten daarom met gepaste terughoudendheid beoordeeld worden. De patiëntkarakteristieken kwamen in beide groepen niet helemaal overeen, vooral omdat in de met alemtuzumab behandelde groep meer patiënten een retransplantatie hadden ondergaan. Deze patiënten waren na een vorige transplantatie reeds behandeld met ATG en het voorkómen van herhaalde blootstelling aan ATG was de belangrijkste reden om te behandelen met alemtuzumab. Deze disbalans zou echter in het nadeel van de alemtuzumab behandelde patiënten kunnen zijn, omdat patiënten die een retransplantatie ondergaan vaak geïmmuniseerd zijn en daarom lastiger zijn te behandelen voor hun rejectie. Een andere beperking van het onderzoek was de relatief korte follow-up van drie maanden. Het T cel depleterende effect van ATG duurt ongeveer 12 maanden en na toediening van alemtuzumab kan dit zelfs nog langer aanhouden. Late bijwerkingen, met name infecties en maligniteiten, kunnen daardoor later dan drie maanden na toediening van ATG of alemtuzumab ontstaan. Tot slot blijkt uit een recent artikel dat het gebruik van alemtuzumab mogelijk geassocieerd is met het ontwikkelen van donorspecifieke anti-HLA antistoffen [217]. Op grond van deze overwegingen biedt onze studie onvoldoende argumenten om alemtuzumab als eerste-lijns behandeling in te zetten bij steroïd-resistente rejectie. Een prospectieve gerandomiseerde en gecontroleerde studie met lange termijn follow-up is hiervoor onontbeerlijk. Desalniettemin kan alemtuzumab de voorkeursbehandeling zijn voor patiënten met steroïd-resistente rejectie waarbij ATG een onaantrekkelijke optie is, bijvoorbeeld na eerdere blootstelling aan ATG of bij aanwezigheid van (cardiovasculaire) comorbiditeit die in het geval van een infusiereactie na toediening van ATG tot problemen zou kunnen leiden.

Zoals reeds vermeld is het T cel depleterende effect van ATG en alemtuzumab langdurig en aspecifiek. Idealiter richten nieuwe monoklonale antistoffen zich alleen op targets op alloreactieve T cellen, zonder langdurige depletie van niet-alloreactieve T cellen, en zonder het vrijkomen van grote hoeveelheden cytokines. Helaas zijn er op dit moment geen studies gaande of gepland met nieuwe monoklonale anti-T cel antistoffen ter behandeling van rejectie. Bij sommige auto-immuunziekten zoals diabetes mellitus worden wel enkele studies gedaan met anti-T cel antistoffen (meestal gericht tegen CD3).

Alhoewel alemtuzumab en ATG dus ongeveer even effectief lijken te zijn ter bestrijding van steroïd-resistente rejectie, is hun werkingsmechanisme niet identiek. De antistoffen in het ATG preparaat binden vooral aan T cel specifieke moleculen zoals CD2, CD3 en CD8, hoewel ATG ook een grote variëteit aan antistoffen gericht tegen NK cellen en endotheelcellen bevat [163,218]. Sommige studies tonen dat ATG geen significante hoeveelheden antistoffen tegen B cel antigenen zoals CD19 of CD20 bevat. ATG heeft echter wel degelijk anti-B cel activiteit, waarschijnlijk direct door depletie van cellen met CD5 of HLA-DR op hun celmembraan, of indirect door het depleteren van CD4+ T cellen en het daarmee elimineren van T cel hulp aan B cellen [16,218]. Alemtuzumab bindt specifiek aan het CD52 molecuul, dat tot expressie komt op T en NK cellen, monocyten, macrofagen, dendritische cellen en B cellen. Deze specifieke interactie met CD52 en directe B cel depletie, zou kunnen bijdragen aan het effect van alemtuzumab bij de behandeling van steroïd-resistente rejectie. De toenemende kennis over de rol die B cellen en antistoffen spelen in acute rejectie heeft de belangstelling voor anti-B cel therapie vergroot. Het aantal klinische studies met anti-B cel therapie bij niertransplantatiepatiënten is echter beperkt.

De resterende hoofdstukken van dit proefschrift beschrijven onderzoeken met als centrale thema het probleem rejectie en diverse aspecten van anti-B cel therapie. In **hoofdstuk 6** staan de uitkomsten beschreven van een gerandomiseerde klinische studie waarin het effect van anti-B cel therapie op de incidentie van acute rejectie werd onderzocht. Een eenmalige gift rituximab of placebo werd toegevoegd aan standaard immuunsuppressie bestaande uit tacrolimus, mycofenolaat mofetil en prednison in een groep van 280 niertransplantatiepatiënten. Hoewel behandeling met rituximab veilig was, leidde het niet tot een vermindering van het aantal afstotingsreacties in de hele onderzoekspopulatie. Bij het opzetten van dit onderzoek hielden we er rekening mee dat de effecten van rituximab niet voor alle patiënten gelijk zouden kunnen zijn. Daarom zijn de geïncludeerde patiënten gestratificeerd naar de aan- of afwezigheid van anti-HLA antistoffen (weerspiegeld door de PRA [panel reactive antigen] waarde) en naar eerste of volgende transplantatie. Wanneer deze strata werden gecombineerd tot een immunologisch laagrisico groep (PRA 6% en eerste transplantatie) en een immunologisch hoogrisico groep (PRA>6% of retransplantatie), was er in de immunologisch hoogrisico groep een duidelijke trend tot minder afstotingsreacties waarneembaar in met rituximab behandelde patiënten, vergeleken met patiënten die placebo kregen toegediend. Opmerkelijk was ook de trend tot minder antistof-gemedieerde rejectie, vooral in met rituximab behandelde hoogrisico patiënten. Deze

studie was echter niet ontworpen voor deze laatste analyse, waardoor voorzichtigheid geboden is bij de interpretatie van de resultaten. Desalniettemin suggereren deze resultaten een beschermend effect van rituximab bij patiënten met een hoog immunologisch risico. Deze bevinding kan mogelijk verklaard worden door depletie van memory B cellen, aangezien deze vaker voorkwamen bij immunologisch hoogrisico patiënten. Echter, uit onderzoek van onder meer onze eigen onderzoekgroep blijkt dat memory B cellen minder gevoelig zijn voor depletie door rituximab dan naïeve B cellen [145,146]. Met een huidige mediane follow-up van 4 jaar, heeft dit gunstige effect van rituximab op de incidentie van rejectie niet geleid tot een verbetering van transplantaat- of patiëntoverleving. Bovendien is het nog onduidelijk of behandeling met rituximab ook leidt tot een verminderde vorming van donorspecifieke anti-HLA antistoffen (DSA). Het routinematig meten van deze DSA's was geen onderdeel van de huidige studie en was daarom niet meegenomen als secundair uitpunt. Momenteel worden bloedmonsters die gedurende de studie verzameld zijn onderzocht op de aanwezigheid van DSA's op verschillende tijdstippen na transplantatie. Gezien de bestaande onzekerheden is het nog te vroeg om behandeling met rituximab toe te voegen aan de standaardbehandeling voor immunologisch hoogrisico patiënten.

Een aspect dat speciale aandacht behoeft is de dosis en intensiteit van de behandeling met rituximab. Het uitblijven van een effect in de gehele onderzoekspopulatie, rechtvaardigt mogelijk een intensiever behandelingsschema, bijvoorbeeld een hogere dosering of een herhalingsdosis. In eerder onderzoek is aangetoond dat een eenmalige toediening van rituximab leidt tot volledige B cel depletie in het perifere bloed, maar niet in de secundaire lymfoïde organen en dat meerdere doses rituximab nodig zijn om B cellen uit lymfeklieren te verwijderen [146,219]. Echter, wij zagen dat na een eenmalige gift rituximab de depletie van B cellen in het perifere bloed langer dan 12 maanden aanhield. Een intensiever behandelingsschema zou mogelijk tot nog langere depletie van B cellen in het perifere bloed kunnen leiden, waardoor het risico op infecties of maligniteiten zou kunnen toenemen. Aan de andere kant kan ook met doseringen lager dan de standaarddosering van 375 mg/m^2 B cel depletie in het perifere bloed worden bereikt. Zo werd aangetoond dat doseringen van 15-35 mg/m^2 leidden tot langdurige B cel depletie in het perifere bloed en de milt [220]. Wat het meest optimale doseringsschema is, is op dit moment dus nog niet duidelijk.

De incidentie van infecties en maligniteiten was niet verhoogd bij met rituximab behandelde patiënten, ondanks de langdurige B cel depletie in het perifere bloed en het

optreden van een tijdelijke neutropenie bij 25% van de patiënten. De absolute incidentie van infecties was hoog (75-80% van de patiënten had één of meerdere infecties doorgemaakt in de eerste 24 maanden na transplantatie) en dat kan een additioneel effect van rituximab hebben gemaskeerd. We vonden echter ook geen verschil in het type of de ernst van de infecties, noch een verschil in ziekenhuisopname en opnameduur. Het uitblijven van een verhoogde incidentie van infecties is opmerkelijk, gezien de rol die B cellen spelen in de afweer tegen micro-organismen. Aangeboren afwijkingen aan B cellen leiden vaak tot agammaglobulinemie, wat zich kenmerkt door recidiverende (bovenste) luchtweginfecties [221]. Een mogelijke verklaring voor onze bevindingen is de gedeeltelijke depletie van B cellen. Immers, zoals hier boven vermeld leidt een eenmalige toediening van rituximab tot B cel depletie in het perifere bloed, maar niet in de secundaire lymfoïde organen [146]. Bovendien is bij andere studies met rituximab in niertransplantatiepatiënten geen daling van immunoglobulineconcentraties waargenomen. Al met al lijkt eenmalige toediening van rituximab een veilige behandeling.

Rituximab leidt tot effectieve depletie van B cellen in het perifere bloed, zoals getoond in hoofdstuk 6. Echter in lymfeklieren blijft een populatie B cellen achter met andere functionele eigenschappen [146]. De vraag of rituximab ook in staat is B cel infiltratie in de nier te verminderen (gedurende rejectie), is onderzocht in **hoofdstuk 7**. Om deze vraag te beantwoorden is de mate van B cel infiltratie gescoord in biopten waarin acute rejectie volgens de Banff classificatie zichtbaar was. Biopten van patiënten die behandeld werden met de standaard immunosuppressiva, bestaande uit tacrolimus, mycofenolaat mofetil en prednison, toonden een wisselende mate van B cel infiltratie. Echter, in biopten van met rituximab behandelde patiënten, was er nagenoeg geen B cel infiltratie. Er werd geen verschil gezien in de ernst van de tubulitis, arteritis of de uitgebreidheid van het cellulaire infiltraat in beide groepen. In voorgaande studies was de aanwezigheid van B cellen in het infiltraat tijdens een periode van rejectie geassocieerd met een hogere kans op steroïdresistentie en transplantaatverlies. In ons cohort was de afwezigheid van B cellen in het infiltraat echter niet voorspellend voor een betere respons op behandeling of verbeterde uitkomsten. Deze bevinding trekt de pathofysiologische rol van B cellen tijdens de acute fase van een rejectie in twijfel. Onze data suggereren dat de aanwezigheid van B cellen in het transplantaat ten tijde van acute rejectie meer als een epifenomeen kan worden beschouwd, dan als een bijdragende pathologische factor.

Activatie van B cellen kan leiden tot de afgifte van een scala aan cytokines [179]. Behandeling met rituximab bij lymfoompatiënten die hoge aantallen B cellen hebben, is berucht om een klinisch syndroom van koorts, rillingen en hypotensie. Dit wordt veroorzaakt door een massale afgifte van cytokines [137]. In **hoofdstuk 8** is onderzocht of een vergelijkbare uitstoot van cytokines optreedt na infusie van rituximab aan niertransplantatiepatiënten. We vonden inderdaad een tijdelijke verhoging van de concentraties van cytokines in het serum, vooral van IL-10 en MIP-1β 2 en 4 uur na start van de infusie, maar geen van de patiënten ervoer het bovenbeschreven klinische syndroom. Bij aanvullende in vitro experimenten waarin rituximab werd toegevoegd aan verschillende populaties PBMC's (perifere bloed mononuclaire cellen) vonden we dat MIP-1β alleen werd afgegeven als rituximab werd toegevoegd aan een co-cultuur van B cellen en NK cellen. Intracellulaire kleuring van MIP-1β liet zien dat de NK cellen en niet de B cellen het MIP-1β produceerden. Incubatie van PBMC's met rituximab-F(ab')2 leidde niet tot een verhoogde afgifte van MIP-1β, wat erop duidt dat deze cytokine afgifte door de NK cellen gemedieerd werd via de Fc-receptor .

De tijdelijke toename van de concentratie van IL-10 en MIP-1β kan klinische relevant zijn, omdat deze cytokines het immuunsysteem kunnen moduleren en daarmee de uitkomst na transplantatie kunnen beïnvloeden. IL-10 wordt in het algemeen als een anti-inflammatoir cytokine beschouwd [189]. De rol van MIP-1β lijkt meer ambivalent te zijn. In muizenmodellen is het een krachtige chemotactische factor voor regulatoire T cellen, maar in andere modellen zorgt MIP-1β, uitgescheiden door NK of B cellen, via de CCR5 chemokine receptor voor rekrutering van monocyten, T cellen en dendritische cellen naar de plaats van ontsteking [190,191]. Daarom kan de afgifte van IL-10 en MIP-1β tegengestelde effecten hebben op het immuunsysteem. De klinische consequenties van deze bevindingen zijn dus nog onduidelijk.

In de laatste studie van dit proefschrift, beschreven in **hoofdstuk 9**, wordt de aandacht gevestigd op de interactie van B cellen met T cellen. Tegenwoordig is het duidelijk dat er geen absolute differentiatie van T cellen is, maar dat T cel subsets aanzienlijke plasticiteit vertonen, afhankelijk van een scala aan signalen [233]. Th17 cellen zijn een relatief nieuwe subpopulatie die wordt gekenmerkt door de productie van bepaalde cytokines, te weten IL-17 en IL-21. Th17 cellen spelen een rol in de bescherming tegen extracellulaire pathogenen, in diverse auto-immuunziekten en volgens recente data ook in acute rejectie. Gebaseerd op voorgaand onderzoek was onze hypothese dat B cellen betrokken zijn bij een Th17 respons. Om deze hypothese te testen, hebben we PBMC's

verkregen bij een subset van patiënten uit de rituximabstudie. We hebben deze PBMC's ex vivo gestimuleerd en maten hierna in het kweekmedium diverse cytokines afkomstig uit monocyten en T cellen. Alhoewel bij alle patiënten de productie van de meeste cytokines was onderdrukt, vergeleken met de periode voor de transplantatie, werd er geen additioneel effect van rituximab aangetoond, ondanks een uitgesproken B cel depletie. Deze bevindingen wijzen er op dat B cellen niet noodzakelijk zijn voor de inductie van een Th17 respons, wat in tegenspraak is met de bevindingen uit eerder onderzoek waarin een afname in de Th17 respons gezien werd na toevoeging van rituximab in vitro aan normale PBMC's [5]. Echter, wij voegden rituximab niet in vitro, maar in vivo toe; twee situaties die fundamenteel van elkaar verschillen. Een interessante bevinding in deze studie is dat ex vivo de IL-17 productie niet onderdrukt was, noch op één dag, noch op één maand na transplantatie, ondanks het gebruik van bovengenoemde immuunsuppressiva. Indien een pathologische rol van IL-17 en Th17 cellen bij niertransplantaat afstoting in de toekomst bewezen wordt, zullen andere immuunsuppressiva noodzakelijk zijn om deze reactie te onderdrukken. Behandeling met rituximab zal daar waarschijnlijk geen positieve bijdrage aan kunnen leveren.

Het doel van dit proefschrift was om de veiligheid en effectiviteit van nieuwe behandelmethoden bij niertransplantatie te onderzoeken. Wij vonden dat ATG-F niet in staat was om de incidentie van delayed graft function te verminderen, terwijl het wel geassocieerd was met een verhoogde incidentie van serieuze bijwerkingen. Daarom is een andere aanpak noodzakelijk om de incidentie van delayed graft function te verminderen. Bij patiënten met een steroïd-resistente rejectie zal ATG-Thymoglobuline de eerstelijns behandeling blijven, alhoewel alemtuzumab effectief en veilig was en daarom een rationeel alternatief kan zijn in aanwezigheid van anti-ATG antistoffen of in gevallen waarin behandeling met ATG te risicovol wordt geacht. Onze studie waarin rituximab is toegediend om B cellen te depleteren, toonde dat alleen immunologisch hoogrisico patiënten mogelijk baat hebben van inductietherapie met een eenmalige gift rituximab. Bij immunologisch laagrisico patiënten konden wij geen effect van rituximab op de incidentie van rejectie vinden. Ondanks een verhoogde incidentie van neutropenie was behandeling met rituximab veilig en leidde het niet tot een verhoogde incidentie van infecties of maligniteiten. Inductietherapie met rituximab was in staat om effectief B cellen te depleteren in het perifere bloed en ten tijde van rejectie in nierweefsel. Voor het transplantaat had dit laatste echter geen bewezen positief effect. In vivo kon een klein

maar duidelijk effect van rituximab worden aangetoond op de serumconcentraties van IL-10 en MIP-1β, alhoewel de gevolgen hiervan momenteel onduidelijk zijn. Ex vivo werd MIP-1β afgegeven door NK cellen wanneer rituximab werd toegevoegd aan een co-cultuur van B cellen en NK cellen. Deze afgifte was afhankelijk van de interactie tussen rituximab en de Fc-receptoren van NK cellen. Tot slot hebben in vitro experimenten aangetoond dat anti-B cel therapie niet in staat is om een Th17 respons te onderdrukken.

De studies in dit proefschrift hebben bijgedragen aan een beter begrip van de effecten van anti-T cel therapie met ATG-F ter preventie van delayed graft function en de waarde van alemtuzumab bij de behandeling van steroïd-resistente rejectie. Bovendien heeft de klinische studie met rituximab samen met de aanvullende experimenten een beter beeld gegeven van de rol die B cellen spelen in de alloimmuun respons. Deze onderzoeken hebben laten zien dat anti-B cel therapie in bepaalde gevallen een nieuwe behandelwijze kan worden bij niertransplantatiepatiënten.

Chapter 12

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Chapter 13

List of publications

List of publications

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21. van den Hoogen M, Steenbergen E, Florquin S, Baas M, Hilbrands L. Intragraft B cells during renal allograft rejection: effect of rituximab induction therapy. Submitted

Chapter 14

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Chapter 15

Curriculum Vitae

Martijn van den Hoogen werd op 22 juli 1981 geboren te Apeldoorn. In 1999 behaalde hij zijn VWO-diploma aan de Koninklijke Scholengemeenschap te Apeldoorn. Dat zelfde jaar begon hij zijn studie geneeskunde aan de Katholieke Universiteit Nijmegen. Eind 2005 behaalde hij zijn artsexamen. Na een korte voortzetting van zijn onderzoeksstage op de afdelingen hematologie en nefrologie (onderwerp: bloedingen na nierbiopsieën, begeleiders Dr. L.B. Hilbrands en Dr. I.R.O. Nováková) begon hij in maart 2006 aan zijn opleiding tot internist in het UMC St Radboud te Nijmegen (opleider Prof. dr. J.W.M. van der Meer, later gevolgd door Prof. dr. J. de Graaf). Kort nadien zette hij zijn opleiding voort in het Jeroen Bosch Ziekenhuis te Den Bosch (opleider Dr. P. Netten) Gestimuleerd door Dr. J. Beutler en aangemoedigd door de opleider zocht hij contact met de afdeling nefrologie in het UMC St Radboud om daar wetenschappelijk onderzoek te doen. In december 2007 keerde hij terug naar het UMC St Radboud om zijn opleiding tot internist te combineren met wetenschappelijk onderzoek naar de effecten van ATG en rituximab bij niertransplantatiepatiënten (supervisoren Prof. dr. A.J. Hoitsma en Prof. dr. L.B. Hilbrands). Deze onderzoeken hebben uiteindelijk geleid tot dit proefschrift.

In september 2012 startte hij met zijn enkelvoudige differentiatie nefrologie (opleider Prof. dr. J.H.M. Berden, later gevolgd door Prof. dr. J.F.M. Wetzels). Per maart 2014 was hij internist en op 1 september 2014 voltooide hij zijn enkelvoudige differentiatie nefrologie. Hierna is hij twee maanden werkzaam geweest op de afdeling nefrologie van het Radboudumc. Per 1 november 2014 is hij werkzaam als internist-nefroloog in het Erasmus MC te Rotterdam.

Sinds november 2009 deelt hij zijn leven met Katarzyna Grygiel.

Nawoord

Tijdens het bezoek aan het Amerikaanse Transplantatie Congres in Boston in juni 2012 liep ik langs een wand waar nabestaanden herinneringen hadden opgeschreven aan familieleden die na hun dood hun organen voor donatie hadden afgestaan. De tekst van één van die gedenktekens heeft mij erg gegrepen en ik heb deze tekst op de volgende pagina overgenomen, uit respect voor alle orgaandonoren en hun nabestaanden.

To remember me

Give my sight to the man who has never seen a sunrise, a baby's face or love in the eye of a woman.

Give my heart to a person whose own heart has caused nothing but endless days of pain.

Give my blood to the teenager who was pulled from the wreckage of his car,
so that he might live to see his grandchildren play.

Give my kidneys to the one who depends on a machine to exist from week to week.

Take my bones, every muscle, every fiber and nerve in my body and find a way to make a crippled child walk.

If you must bury something, let it be my faults, my weaknesses and all prejudice against my fellow man.

Give my sins to the devil.

Give my soul to God.

If by chance, you wish to remember me, do it with a kind deed or word to someone who needs you.

If you do all I have asked I will live forever.